PRACTICAL HAEMATOLOGY

BY

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PREFACE TO THE SECOND EDITION

IMPORTANT advances have been made in our knowledge of blood diseases since the first edition of *Practical Haematology* was published, and the laboratory techniques required for the investigation of patients have correspondingly increased in number and complexity. In preparing a new edition I have revised and virtually rewritten the whole work in an attempt to make it an up-to-date and practical laboratory guide. It is hoped that it will prove useful to hospital pathologists, postgraduate students of clinical pathology and senior laboratory technicians in their day-to-day routine investigation of haematological and transfusion problems as well as serving as a source of information on methods of investigation which are less often required

As in the previous edition, I have not attempted to describe or illustrate the appearances of normal or pathological blood cells when stained by Romanowsky dyes To do this adequately would have increased the size and price of the book beyond what was intended. I have, however, retained a brief description of the appearances of blood cells which have been stained supravitally and I have added illustrations of Heinz bodies and siderotic granules

I have been fortunate in the help I have received from my colleagues in the Department of Pathology of the Postgraduate Medical School of London Dr W R Pitney and Dr J C White have given me invaluable advice on methods of investigating the haemorrhagic disorders and abnormal haemoglobins, respectively, and Professor E. J. King and Dr I D P Wootton have helped me greatly in the section on haemoglobin estimation I am also much indebted to Dr. J C Crookston, who read the complete typescript and made many pertinent suggestions, and to other colleagues who have read the proofs in whole or in part I should also like to thank Miss R Klein for the photomicrographs, the Department of Medical Illustration of the Postgraduate Medical School of London for the photographs of apparatus, and Mr F G Saunders for drawing certain of the figures

I am glad to be able to record my indebtedness to Drs Rosemary Biggs and A S Douglas and the Editor of the *Journal of Clinical Pathology* for permission to reproduce Figure 37, to Drs L S Sacker and B E C Nordin and the Editor of the *Lancet* for permission to

reproduce Figure 13; to Sir Lionel Whitby and Dr. C. J. C. Britton for permission to reproduce Figures 12 and 40 from their *Disorders of the Blood*; and to Drs J. C. White and G. H. Beaven and Miss M. Ellis for permission to reproduce Figures 28 and 29.

J. V. DACIE.

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CHAPTER 1

COLLECTION OF BLOOD AND NORMAL VALUES

COLLECTION OF BLOOD FROM PATIENTS

VENOUS blood is preferred for most haematological examinations, but peripheral samples can be almost as satisfactory for some purposes if a free flow of blood is obtained.

Venous Blood

This is best withdrawn from an antecubital vein by means of a dry syringe Alternatively, a needle to which about 5 cm of rubber tubing have been attached can be used in patients who have good veins. The needles should not be too fine or too long. Those of 19 or 20 S.W.G are suitable for use with syringes If a syringe is not being used, they should be of larger bore, e g., 16 S W.G., with these needles 100 ml or more of blood can be easily withdrawn. Short needles with shafts about 15 mm long are particularly suitable for use with children

Ideally, congestion should be completely avoided, in practice a tourniquet is usually necessary—it can often be loosened once the needle has been inserted into the vein It is likely that complete stasis for as little as 30 seconds causes some haemoconcentration (26) The piston of the syringe should be withdrawn slowly and no attempt made to withdraw blood faster than the vein is filling After detaching the needle, the blood should be delivered carefully from the syringe into a container, and if it is desired to prevent coagulation it should be promptly and thoro[illegible]

If b[illegible]

the bl[illegible]

volume of blood on to a waxed watch glass and to make the films (or collect a measured sample of blood for a platelet count) from this (see pp 37 and 49)

Haemolysis can be avoided or minimized by using clean apparatus, withdrawing the blood slowly, not using fine needles, delivering the blood gently into the receiver and avoiding frothing during the withdrawal of the blood and subsequent mixing with the anticoagulant

Blood collected in order to obtain serum should be delivered into sterile tubes or screw-capped bottles and allowed to clot undisturbed for 1 to 2 hours at 37°C When the blood has firmly clotted and the clot has started to retract, the sample may be left in a refrigerator

overnight at 4°C., so that clot retraction may become complete under conditions unfavourable for the growth of bacteria. If the clot shows no sign of retraction, it may be gently detached from the walls of the container by means of a platinum wire or sealed Pasteur pipette. If it is roughly treated haemolysis is certain to follow.

When serum is required with the minimum of delay or when both serum and cells are required, as in the investigation of certain types of haemolytic anaemia, the sample can be defibrinated. This is simply performed by placing the blood in a receiver such as a conical flask containing a central glass rod on to which small pieces of glass capillary have been fused (see p. 222). The blood is whisked around the central rod by moderately rapid rotation of the flask. Coagulation is usually complete within 5 minutes, most of the fibrin collecting upon the central rod. When fibrin formation seems complete, the defibrinated blood may be centrifuged and serum obtained quickly and in relatively large volumes Blood defibrinated in this way should not undergo any appreciable degree of haemolysis. The morphology of the red cells and the leucocytes is well preserved. Defibrinated blood is thus a good source of leucocytes for buffy-coat preparations and for demonstrating the L.E.-cell phenomenon.

When cold agglutinins are known to be present in high concentrations it is best to bring the patient to the laboratory and, using a needle connected to a short length of rubber tubing, to collect blood into containers previously warmed to 37°C. When filled they should be promptly replaced in the 37°C water-bath. In this way it is possible to obtain serum free from haemolysis even when cold antibodies are present capable of causing agglutination at temperatures as high as 30°C. The blood must be left undisturbed at 37°C until the serum has separated. When the clot has retracted and clear serum has been expressed, the serum is removed by Pasteur pipette, transferred to a tube warmed by being allowed to stand in the water-bath and then rapidly centrifuged so as to rid it of any suspended red cells

Anticoagulants

Several good anticoagulants are available for use in clinical haematology: ammonium and potassium oxalate mixture, Sequestrene, sodium citrate, and heparin

Ammonium and Potassium Oxalate Mixture (Heller and Paul (11))

The mixed salts (six parts of ammonium oxalate to four parts of potassium oxalate) are used at a concentration of 2 mg per 1 ml. of blood. The mean corpuscular volume is unaltered and little haemolysis caused. Such blood may be used for haematocrit and haemoglobin estimation, and for red-cell and leucocyte counts, and the plasma

for the estimation of bilirubin and prothrombin time. Films are satisfactory only if they are made without delay. If the blood is allowed to stand for more than a few minutes before films are made, definite progressive changes take place in the morphology of the corpuscles. The red cells start to crenate and the leucocytes undergo important changes. The cytoplasm of the granulocytes becomes vacuolated and ingested oxalate crystals may be seen. The nuclei of lymphocytes and monocytes undergo bizarre changes in shape; lobation or budding takes place and pathological forms may be simulated. Mistakes may be made unless this type of change is appreciated.

Sequestrene (EDTA)

The disodium salt of ethylenediamine tetra-acetic acid is a powerful anticoagulant which is more effective weight for weight than sodium citrate (5, 9, 19). It is a useful alternative to potassium and ammonium oxalate mixture and has some advantages It is effective at a concentration of 1·0 to 2·0 mg per ml of blood. Care must be taken, however, to ensure by repeated inversions of the container that the Sequestrene is thoroughly mixed in the blood added to it. At this concentration it does not appear to affect the packed cell volume According to Hadley and Weiss (9a) the dipotassium salt of EDTA, because of its much higher solubility, is an even more satisfactory anticoagulant than the disodium salt.

Sequestrene is non-toxic—it can be used as an anticoagulant in blood transfusions—and has less effect than oxalate mixture on blood *in vitro*. It is thus preferable to oxalate mixture in routine blood examinations if for any reason blood films cannot be made immediately after collection. Even if the blood is allowed to stand on the bench for 2 to 3 hours before films are made, leucocyte and red-cell morphology is well preserved and good staining can be obtained Reliable total red-cell and leucocyte counts can be carried out on blood which has been allowed to stand for as long as 12 hours. Sequestrene most effectively prevents the clumping of platelets *in vitro*, and for this reason platelet counts can also be carried out quite reliably on Sequestrene-treated blood which has been allowed to stand for several hours Its use also enables a rough estimate to be made of the platelet count by inspection of a stained film. When other anticoagulants are used—in which platelets clump rapidly—any such estimates are unreliable.

Trisodium Citrate

A 3·8% aqueous solution is the anticoagulant of choice in coagulation studies Nine parts of blood are added to one part of the sodium citrate solution and immediately well mixed with it. Sodium citrate is also the anticoagulant most widely used in the estimation of the sedimentation rate (E S R).

Heparin

This may be used at a concentration of 0·1 to 0·2 mg per ml. of blood. Heparin is an effective anticoagulant and does not alter corpuscular size; it is the best dry anticoagulant when it is important to reduce to a minimum the chance of haemolysis occurring after blood has been withdrawn. However, heparinized blood should not be used for making blood films as it gives a faint blue coloration of the background when the films are stained by Romanowsky dyes. Heparin is the best anticoagulant to use for osmotic fragility tests; otherwise it is inferior to mixed oxalates or Sequestrene for general laboratory use.

Mode of Action of Anticoagulants

Ammonium and potassium oxalates, Sequestrene and sodium citrate remove calcium which is essential for coagulation. Calcium is either precipitated as insoluble oxalate (crystals of which may be seen in oxalated blood) or bound in an un-ionized form. Heparin inhibits coagulation in a different way; it is thought to have the power of neutralizing thrombin in the presence of a co-factor located in the albumin fraction of serum. Ammonium and potassium oxalates are poisonous and are for laboratory use only. Sodium citrate (preferably the disodium salt), Sequestrene and heparin can be used to render blood incoagulable before transfusion.

Storage of Blood before Estimations are Performed

Regardless of the anticoagulant, certain changes take place when blood is allowed to stand *in vitro*. They can be minimized by keeping the samples in a refrigerator at 4°C. The red cells start to swell, with the result that the mean corpuscular volume increases, osmotic fragility and prothrombin time slowly increase and the sedimentation rate decreases, the leucocytes gradually autolyse (6). Haemoglobin remains unchanged for days. However, these changes take place slowly, and for many purposes blood may be safely allowed to stand overnight in a refrigerator if precautions against freezing are taken. Nevertheless, it is best to count leucocytes and estimate the sedimentation rate within 6 hours of collection. As already mentioned, Sequestrene is for some purposes the best anticoagulant to use if the estimations cannot be carried out shortly after the blood is collected. The advisability of making films at once has already been stressed.

The importance of effectively mixing blood after collection, particularly if it has been stored and is cold an[illegible] nnot be over-emphasized. If cold, the blo[illegible] uld first [illegible] to warm up to room temperature, then [illegible] erably [illegible], for at least 2 minutes. The difficulty [illegible] ored b[illegible] ong point in favour of performing bloo[illegible] out d[illegible]

“Capillary” (Pe[illegible]heral) I[illegible]

This can [illegible] from the h[illegible] fro[illegible]

is essential, and only the very gentlest squeezing is permissible; ideally, large drops of blood should exude slowly but spontaneously. If it is necessary to squeeze firmly in order to obtain blood, the results are unreliable. If the poor flow is due to the part being cold and cyanosed, too high figures for red-cell counts, haemoglobin content and leucocyte counts are usually obtained

The discrepancies between peripheral and venous samples are more marked if the ear-lobe rather than the finger is chosen as the site for puncture (2, 13) However, if the ear is rubbed well with a square of lint until it is pink and warm, a good spontaneous flow of blood is obtained from most patients if glass capillaries are used as prickers (see below). Under these circumstances the figures for red-cell counts, haemoglobin content and leucocyte counts closely approximate to those of venous blood. For "screening" haematology the author prefers to obtain blood from the ear rather than the finger for the following reasons: an ear-lobe puncture is much less painful, a fresh sterile glass capillary pricker can be used for each patient, and because relatively large volumes of blood can be readily obtained if the ear is skilfully punctured.

Ear-lobe puncture is carried out as follows. The ear must be rubbed with lint until warm It is then pricked to a depth of 2 to 3 mm. with a sterile glass capillary pricker* or needle The glass capillary is placed against the most dependent part of the lobe of the ear and inserted by gentle rotation The first few drops should be wiped away and the sample collected when the blood is flowing spontaneously, usually in about half a minute A separate glass capillary pricker is used for each patient. These are made from wide glass capillaries partially pulled out (Fig. 1) With a little practice it is possible to break them so that the cut end is smooth and perpendicular

Glass capillaries are too fragile for use in pricking the finger. A sterile Hagedorn or similar needle should be used instead It is desirable to use a fresh needle for each patient, for only by flaming the needle between each puncture—which soon results in blunting it—can it be effectively sterilized Dipping in alcohol is insufficient to exclude the possibility of transmitting serum hepatitis from one patient to the other

Heel Blood

Satisfactory samples can be obtained in infants by a deep puncture using a stout needle, but only if the heel is really warm—it may be necessary to bathe it in hot water Even so, skin-prick samples give haemoglobin values somewhat higher than do venous samples.

* Glass capillary prickers for obtaining blood from the finger were described in 1912 by Sir Almroth Wright in his *Handbook of the Technique of the Teat and Capillary Glass Tube* They were in routine use in the pathological and biochemical laboratories of King's College Hospital, London, when I was a student

Particularly is this true of the first day of life, when the average difference is as much as 2·0 g. per 100 ml. (15).

DIFFERENCES BETWEEN "CAPILLARY" AND VENOUS BLOOD

It is not quite clear whether the packed cell volume (haematocrit value), red-cell count and haemoglobin content of venous blood and capillary blood are the same, even if the latter is freely flowing. Although the results of Price-Jones, Vaughan and Goddard (18) did not

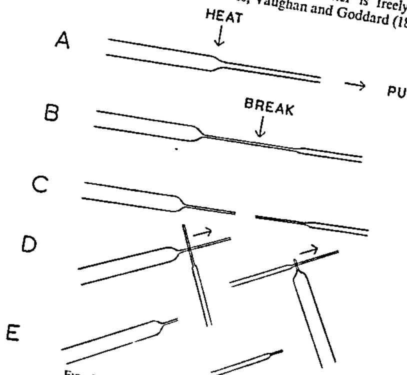


FIG 1 MAKING GLASS CAPILLARY PRICKERS
Glass tubing of 6–7 mm bore is pulled out so as to form a stout capillary (A) Next, a more slender capillary (B) is made by heating the shoulder of the tubing The fine capillary is then broken in the middle (C) Downward pressure combined with movement along the axis of the capillary (D) will cause a vertical break 3–5 mm from the shoulder of the tubing (E) The other end of the fine capillary can be shortened in a similar way

reveal any certain differences in normal adults, this may not be always true Strauss and Burchenal (21), in a survey of 80 patients under treatment for pernicious anaemia, found that their red-cell counts and haemoglobin levels averaged 5% higher in capillary than in venous blood, a difference which was statistically significant. In infants, as already mentioned, even greater differences seem to exist, at least in the neonatal period (15)

It is likely that freely flowing blood obtained by skin puncture is more nearly arteriolar in composition than capillary. Indeed, the haematocrit value, red-cell count and haemoglobin content of true capillary blood are probably significantly less than those of venous blood (8). The platelet count appears to be higher in venous than in capillary blood—this may, however, be due to adhesion of platelets at the site of the skin puncture. Leucocyte counts are probably identical, but only if the peripheral blood is freely flowing—if the ear is cold, the capillary count may be much higher than the venous count (13) The osmotic fragility of venous blood is significantly greater than that of peripheral blood, due probably to the lowered pH and reduced oxygen tension in the venous sample.

NORMAL VALUES IN HAEMATOLOGY

It is extremely difficult to state the normal limits of haematological values; the observed ranges are considerable, and age and sex determine important differences. In addition, variation in technique may explain differences between some observers' figures; particularly is this true of such estimations as the counting of platelets and the whole-blood coagulation time.

The borderline between health and ill-health is indefinite, so it is with haematological values, for the normal and abnormal undoubtedly overlap. For instance, a value well within the recognized "normal range" may be definitely pathological in a particular subject, e g , a total leucocyte count of 10,000 cells per c mm. is abnormal for a man whose count usually ranges between 4,000 and 6,000 per c.mm

The data given in Table 1 are derived from various sources (7, 15, 17, 18, 24, 25) Other references are given in later chapters when the actual techniques are described The figures given are believed to cover at least 95 % of healthy subjects

TABLE 1

NORMAL HAEMATOLOGICAL VALUES
VARIATION IN HEALTH

Red cells	
Men	4 5–6 5 million per c mm
Women	3 9–5 6 " " "
Infants (full-term, cord blood)	4 0–5 6 " " "
Children, 1 year (mean)	4 5 " " "
Children, 10 years (mean)	4 7 " " "
Haemoglobin	
Men	13 5–18 0 g per 100 ml
Women	11 5–16 4 g " " "
Infants (full-term, cord blood)	13 6–19 6 g " " "
Children, 1 year (mean)	11 2 g " " "
Children, 10 years (mean)	12 9 g " " "

Packed cell volume (haematocrit value)

Men	40–54%
Women	36–47%
Infants (full-term, cord blood)	44–62%
Children, 1 year (mean)	35%
Children, 10 years (mean)	37·5%

Mean corpuscular volume (M.C.V.)

Adults	*76–96 c μ*

Mean corpuscular haemoglobin (M.C.H.)

Adults	*27–32 μμg*

Mean corpuscular haemoglobin concentration (M.C.H.C.)

Adults	*32–36%*

Mean corpuscular diameter (dry films)

Adults	6·6–7.7 μ

Reticulocytes

Adults	0.2–2.0%
Infants (full-term, cord blood)	2–6%

Leucocytes

Adults	4,000–10,000 per c mm
Infants (full-term, at birth)	10,000–25,000 ,, ,,
Infants (1 year)	6,000–18,000 ,, ,,
Childhood (4–7 years)	6,000–15,000 ,, ,,
Childhood (8–12 years)	4,500–13,500 ,, ,,

Differential leucocyte count

Adults	Neutrophils	40–75%	2,500–7,500 per c mm
	Lymphocytes	20–50%	1,500–3,500 ,, ,,
	Monocytes	2–10%	200– 800 ,, ,,
	Eosinophils	1–6%	40– 440 ,, ,,
	Basophils	1%	15– 100 ,, ,,

Platelets 150,000–400,000 per c mm

[illegible] 0–7 min

[illegible] 5–11 min

[illegible] [illegible]uick)) 10–14 sec

[illegible] 0–40%

[illegible] 200–400 mg per 100 ml

Osmotic fragility [illegible]

% NaCl	[illegible]	[illegible]
0.20	100	91–100
0.30	97–100	80–100
0.35	90– 99	72–100
0.40	50– 90	65–100
0.45	5– 45	54– 96
0.50	0– 5	36– 88
0.55	0	5– 70
0.60	0	0– 40
0.65	0	0– 19
0.70	0	0– 9
0.75	0	0– 2
0.80	0	0
0.85	0	0

Median corpuscular fragility (M.C.F.) 0.40–0.445% NaCl

Autohaemolysis (37°C)	24 hours	0–0 5% lysis
	48 hours	0 4–3 5% lysis
Cold-agglutinin titre (4 C)		< 64
Sedimentation rate (method of Westergren)		
Men		3–5 mm in 1 hour, 7–15 in 2 hours
Women		4–7 mm in 1 hour; 12–17 in 2 hours
Sedimentation rate (method of Wintrobe and Landsberg)		
Men		0– 9 mm in 1 hour
Women		0–20 mm in 1 hour
Heterophile (anti-sheep red-cell) agglutinin titre		< 80
Heterophile (anti-sheep red-cell) agglutinin titre (after absorption with guinea-pig kidney)		< 10

PHYSIOLOGICAL VARIATION

Physiological Variation in Haemoglobin Concentration, Haematocrit Value, and Red-Cell Counts (24, 25)

It is well known that there is considerable variation in the red-cell counts and haemoglobin concentrations at different periods of life. At birth the haemoglobin content of the blood is higher than at any period subsequently (Table 1) After the immediate post-natal period the haemoglobin level falls fairly steeply to a minimum of about 11 g per 100 ml at about the fifth to eighteenth month There is, however, considerable individual variation The red-cell count falls less steeply, and the cells become hypochromic Both haemoglobin concentration and red-cell count then rise gradually to almost the adult level by the time of puberty, thereafter the levels in women tend to be significantly lower than those of men (10, 23)

In addition to the permanent effects of age and sex, there seem to be

was 11 %

Physiological Variation in the Total Leucocyte Count (7, 17, 22)

The effect of age is indicated in Table 1; in general, higher levels are observed in childhood. Lymphocytes are the predominant cells from the second week of life until the fifth to seventh year, when they give way to the neutrophil polymorphonuclears. There is no sex difference. People differ considerably in their leucocyte counts, but each tends to maintain a relatively constant picture despite irregular oscillation during which variations up to 50% of the count are said to occur. The minimum count is found in the morning with the subject at rest; the maximum in the afternoon. "Random activity" may raise the count slightly; strenuous exercise causes increments of up to 30,000 cells per c mm., chiefly due, it is thought, to liberation into the blood stream of neutrophils formerly sequestered in shut-down capillaries. Large numbers of lymphocytes from lymphatic channels also probably enter the blood stream during strenuous exercise

Adrenaline injection also causes an increase in the leucocyte count; here, too, increases in the numbers of all the major types of leucocytes (and platelets) occur (3). The rise has been thought to be a reflection of the extent of the reservoir of mature blood cells present not only in the bone-marrow and spleen, but also in the other tissues and organs of the body. Emotion may possibly cause an increase in the leucocyte count in a similar way. The effect of ingestion of food is uncertain

The height of the eosinophil count is controlled at least in part by the activity of the adrenal cortex (12, 20), increased adrenocortical activity leading to a fall in the number of circulating eosinophils

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CHAPTER 2

BASIC HAEMATOLOGICAL TECHNIQUES: I

THE TOTAL RED-CELL COUNT

A DIMINISHING but still considerable proportion of a technician's time is taken up in performing red-cell counts. *Unfortunately, this* estimation is fraught with inaccuracies (p. 17), and it is thus particularly important that both the blood counter and the clinician who asks for the count to be undertaken have a clear idea of limitations of the method as usually carried out. *The following technique is recommended*:—

A 1 : 200 dilution of blood is made in formol-citrate solution. This is most conveniently done by washing 0·02 ml. of blood taken into a "haemoglobin" pipette into 4 ml. of diluting fluid contained in a 75 × 10 mm tube After sealing the tube with a tightly fitting rubber bung, the diluted blood should be mixed by hand for at least 2 minutes by tilting the tube through an angle of about 120°, combined with rotation, thus allowing the air bubble to mix the suspension; alternatively, the cell suspension may be mixed in a mechanical mixer (34) (Fig. 2).

The counting chamber, with its cover-glass already in position, should be filled without delay This is simply accomplished with the aid of a Pasteur pipette or a length of stout capillary glass tubing which has been allowed to take up the suspension by capillarity Care should be taken that the counting chamber is filled in one action and that no fluid flows into the surrounding moat The chamber should be left undisturbed for at least 3 minutes for the cells to settle, but not much longer, for drying at the edges of the preparation may initiate disturbing currents which cause movement of the cells after they have settled It is important that the cover-glass should be of a special thick glass and perfectly flat, so that when laid on the counting chamber Newton's diffraction rings can be seen The cover-glass should be of such a size that when placed correctly on the counting chamber the central ruled area or areas lie in the centre of the rectangle to be filled with the cell suspension

The type of counting chamber used and the arrangement of the rulings is largely a matter of taste and availability The visibility of the rulings is as important as the accuracy of calibration.

The cells should be counted, using a 4-mm dry objective and × 6, or × 10 eyepieces (preferably the latter) It is important to count

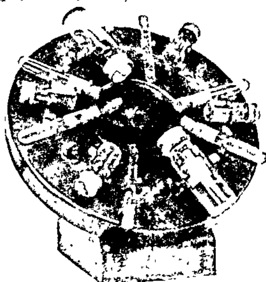


FIG 2 AN ELECTRICALLY DRIVEN CELL-SUSPENSION MIXER (MADE BY TI WORKSHOPS STAFF OF THE POSTGRADUATE MEDICAL SCHOOL OF LONDON)

The motor is a 33⅓ r p m gramophone motor Similar machines (the Matbui suspension mixer) are obtainable from Matburn Ltd, 25 Red Lion Street, London, W C I

as many cells as possible, for the accuracy of the count is increase thereby (see below), 500 cells should be considered the absolute mini mum. With a Neubauer chamber (Figs 3a and 3b), the cells in four o eight horizontal or vertical rectangles of 1 mm × 0 05 mm (80 or 16 small squares) or in five or ten groups of 16 small squares should b counted including the cells which touch the top and right-hand margin and omitting from the count those which touch the bottom and left-han margins of the squares Using a Bürker chamber (Fig. 4), the cell in three (or six) of the 3 mm. × 0 05 mm. narrow rectangles should b counted, including the cells which touch the top and right-han margins.

Calculation (1 . 200 dilution)

Neubauer Chamber. *Number of cells in four 1 mm. × 0 05 mm* rectangles, 0·1 mm. in depth (0 02 c.mm. in volume) = N.

Red-cell count in millions per c mm. $= N \times \frac{1}{0 \cdot 02} \times 200$ (dilution

$= N \times 10{,}000$

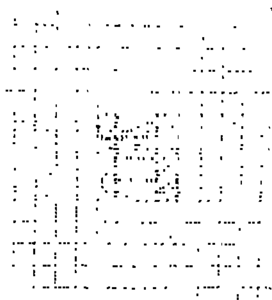


FIG. 3A. NEUBAUER COUNTING CHAMBER
The total ruled area is 3 mm × 3 mm, the central ruled area 1 mm × 1 mm In the central area 16 groups of 16 small squares are separated by triple rulings

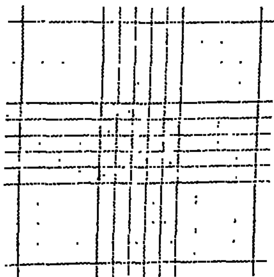


FIG 3B IMPROVED NEUBAUER COUNTING CHAMBER
The central ruled area consists of 25 groups of 16 small squares separated by closely ruled triple lines (which appear as thick black lines in the photograph).

Burker Chamber. Number of cells in three 3 mm. × 0·05 mm. narrow rectangles, 0 1 mm. in depth (0·045 c.mm. in volume) = N.

$$\text{Red-cell count in millions per c.mm.} = N \times \frac{1}{0{\cdot}045} \times 200 \text{ (dilution)}$$
$$= N \times 4{,}440.$$

Red-Cell Diluting Fluid

A solution of 1% (v/v) formalin (40% formaldehyde) in 3% trisodium citrate is recommended. The solution is simple to prepare, it keeps well and does not need to be sterilized. The corpuscles maintain their normal disc-like form and are not agglutinated The cells

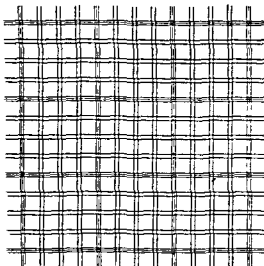


FIG 4 BÜRKER COUNTING CHAMBER

The total ruled area is 3 mm × 3 mm, with triple lines 1 mm apart The narrow rectangles measure 3 mm × 0 05 mm, the squares they enclose have an area of 0 04 sq mm

are well preserved and counts may be performed several hours after the blood has been diluted

Diluting fluids such as those of Hayem and Toison seem to offer no

plete sphering

Notes on Apparatus

Bulb diluting pipettes are not recommended; they are expensive, difficult to calibrate and easily broken. The volumes of blood used are unnecessarily small and the pipettes are difficult to label and handle. In particular, filling the counting chamber so that the exact amount of fluid is delivered from the pipette is an art difficult to master. 0·02-ml. pipettes are relatively inexpensive and easy to calibrate. By the use of 4 ml. of diluting fluid in a tube provided with a tightly fitting rubber bung a suspension easy to label and handle is obtained, and a perfect filling of the counting chamber readily accomplished with the aid of a fine Pasteur pipette or stout glass capillary.

The accuracy of 0·02-ml. pipettes may be checked, after careful cleaning, by filling them to the mark with clean mercury, expelling the mercury and weighing it (43). 0·02 ml. of mercury weighs 272 mg; 0·05 ml. of mercury weighs 680 mg. It is convenient to draw up the mercury to the mark in the pipette by means of an oiled 1-ml. Tuberculin syringe connected to the pipette by a small piece of pressure tubing. The measured column of mercury is then expelled on to a previously weighed watch-glass and weighed in a balance sensitive to a difference of 1 mg.

Normal Range of Red-Cell Counts in Health

Men	4·5–6 5 million per c.mm.
Women	3·9–5 6 ,, ,, ,,
Infants at birth (full-term, cord blood)	4 0–5 6 ,, ,, ,,
Children (1 year) (mean)	4 5 ,, ,, ,,
Children (10 years) (mean)	4 7 ,, ,, ,,

THE ERROR OF THE RED-CELL COUNT

An accurate red-cell count is difficult to accomplish The errors associated with the count are of two main kinds those due to inaccurate apparatus, indifferent technique or unrepresentative nature of the blood counted (" technical " errors) and that due to the distribution of the suspension of red cells in the counting chamber—the " inherent " or " field " error (33) The former errors can be minimized by a careful technique, the latter error can only be overcome by counting large numbers of cells

The distribution of the cells in the counting chamber is of an irregular (random) pattern, even in a perfectly mixed sample However, the pattern of distribution conforms to a definite type. Theoretical considerations indicate that variation between the numbers of red cells which settle in areas similar in size should conform to a mathematical distribution (Poisson series) and that the standard error (σ) of the

distribution of the number of cells in areas of equal size should be given by $\sqrt{m}$ where m is the mean number of cells in the areas concerned (38a, 45). Actual experiments have shown that the distribution of red cells in the counting chamber does, in fact, largely conform to the expected Poisson distribution. In practice, however, $\sigma = 0{\cdot}92\sqrt{m}$, instead of $\sqrt{m}$ (33, 38a), this difference, which is relatively unimportant, is due probably to "overcrowding" in the counting chamber. This type of distribution means that if the counting chamber was filled with a red-cell suspension so that the mean number of cells in an area (say 80 small squares) was 100, then if it were possible to count the number of cells in each of 100 similar areas, in 95 areas the number of cells encountered would range between 80 and 120 ($100 \pm 2\sigma$ ($2 \times \sqrt{100}$) $= 100 \pm 20$); in the remaining five areas the counts would be outside the 80 to 120 range.

Clearly this random distribution has a very important bearing on the accuracy of blood counts, for no amount of mixing will minimize the inherent variation in numbers between area and area.

tice. Assuming for simplicity that the standard error of the number of cells

, $\sqrt{500} = 22$ (approximately), then

of cells encountered would range

These figures represent a red-cell count ranging between 4 56 to 5 44 million per c mm, with chance determining the actual number of cells present within the area selected for the count Fortunately, this very great range in possible results, dependent

The ratio $\frac{\sigma}{m}$ is the coefficient of variation (V) and this expressed as a percentage gives a convenient way of expressing the inherent error of blood counts In the example given above $V = \frac{\sigma}{m} = \frac{22}{500} \times 100 = 4{\cdot}4\%$, and the range $\pm$ 2 V involves an error of $\pm$ 8 8% or $\pm$ 440,000 cells out of a total of 5,000,000.

The *inherent* error in blood counting can be reduced in only one way by counting more cells This may be done in one of two ways, by counting more cells in one preparation or by making successive counts The following calculations (Table II) demonstrate that, in theory, the coefficient of variation (V) of the count varies in proportion

to the square root of the number of cells counted, i.e., if four times the number of cells are counted the coefficient of variation is halved. For example, if the imaginary (ideal) figures given in Table II are studied, it will be seen that 19 out of 20 counts based on the number of cells in 80 small squares will lie within the wide range of 5·12 to 6·08 million per c mm.; however, if the cells in 320 small squares are counted, the range will be considerably narrower—5·36 to 5·84 million per c.mm (*cf.*, Table III)

TABLE II

ERROR OF THE RED-CELL COUNT

No of small squares counted	No. of cells counted (m)	Standard error ($\sqrt{m}$)	Range (95%) ($m \pm 2\sigma$)	Calculated red-cell count (mill /c mm)	Coefficient of variation (V) (%)
80	560	24	512– 608	5 12–6 08	4 3
160	1,120	33	1,054–1,186	5 27–5·93	2·9
320	2,240	47	2,146–2,334	5 36–5 84	2 1
640	4,480	67	4,346–4,614	5 43–5 76	1 5

Table showing how the inherent error of red-cell counting may be reduced by counting larger numbers of red cells

The method of making serial counts and taking the mean has been widely (perhaps unconsciously) used as a means of reducing the error of the red-cell count If a sufficient number of counts are done the truth is likely to reveal itself, not only will the inherent error be reduced by the counting of a large number of cells, but there is a chance that errors in technique will cancel each other out

So far only the inherent error of the red-cell count has been considered. The *technical* errors of the count are also of great importance and may be large. Fortunately, however, much can be done to minimize them The technical errors include bad sampling of the blood due to an inadequate flow from a skin puncture, to prolonged use of a tourniquet or to inefficient mixture of venous blood which has sedimented after collection, inaccurate pipetting and the use of badly calibrated pipettes or counting chambers, inadequate mixing of the red-cell suspension, a bad filling of the counting chamber and careless counting of cells within the chamber

Magath, Berkson, and Hurn (33) have shown that if the number of cells in an area is estimated by visual counting and then checked by accurate counting of a photograph of the counting chamber, the visual estimate is usually too low However, training and care can reduce the discrepancy to small proportions. This point may be illustrated by an actual experiment. Two (experienced) observers were carrying out some serial blood counts using the same suspensions Nevertheless, the figures of one observer were noted to be consistently less than those of the other, the difference in six

successive counts was in the same direction, the counts of Observer B [illegible]

then in agreement.

The results are illustrated by the following figures·

	Observer A	Observer B	"True" value
	135	107	134
	120	108	121
	156	128	143
	124	119	142
Totals (80 small squares)	535	462	540

The experiment was repeated the following day, with much better results (there was no attempt to count 2,000 cells)

	Observer A	Observer B	"True" value
	128	137	124
	145	136	145
	125	126	126
	140	150	143
Totals (80 small squares)	538	549	538

The les[illegible]
of 2,000
theory, n
the coun
necessary degree of concentration (and to be free from interruption) for this length of time. Lack of concentration seems to lead to undercounting. [illegible]

Neubauer chamber it is easiest to count accurately within the centre 1 mm square area of small squares

The summation effect of the technical and inherent components of the [illegible]

millions per c mm ($\pm 2\sigma$) [illegible]

Clearly the errors of blood counting are very considerable. That due to the random distribution of the cells in the counting chamber can be reduced by counting the cells in a larger area, as already mentioned (see Table II), but in ordinary laboratory practice there is rarely time to count carefully the cells in more than 160 small squares—about 1,000 cells in a normal count. The practice of making counts in duplicate is a good one, but does not necessarily increase accuracy: *it is always possible that* the second count will be further from the truth than the first, due to the random distribution of cells. According to White (51), however, it is better to repeat a count using a second chamber and pipette than to count double the number of cells in a single filling of the counting chamber.

As Ponder (38a) points out, the figures of Magath, Berkson and Hurn (2, 33) and Berg (1) illustrate the errors of red-cell counting at their worst, and show the futility of basing results solely on counts made on the cells in 80 small squares (usually 500 cells or less) Ponder (38a) correctly points out that if a sufficient number of cells is carefully counted and if carefully calibrated pipettes and chambers are used, the error is small (not more than $\pm 3\%$), which is not worse than that of many other laboratory estimations. In fact, in the hands of a good, unhurried technician, using good apparatus, the technical errors of the count can be almost abolished However, this does not mean that small differences in red-cell counts necessarily have much significance, even if sufficiently large numbers of cells are counted to reduce the inherent error to small proportions. In Table III are shown the results of a series of daily blood counts carried out on a healthy man by the same observer The results indicate the sort of day-to-day variation which occurs even when counting by ordinary visual means is very carefully carried out. The samples were taken from freely flowing capillary blood. According to Biggs and Allington (3), the day-to-day variation in venous blood is no less than that of capillary blood.

A more recent study of the errors of haematological methods was made by Biggs and MacMillan (4, 5) Their results agree substantially with those of Berkson, Magath and Hurn (2), and with those of Berg (1). They point out the importance of personal bias in the performance of counts, and that inaccuracy results from foreknowledge of what the result should be; e.g., counts made on five samples of the same blood will more closely correspond if the blood counter knows that the five samples are part of a single large sample In performing a count on a single filling of a counting chamber, there is often an unconscious tendency to minimize the differences in the cell counts from successive areas; thus the number of cells in the first area counted may determine

TABLE III

THE RED-CELL COUNT OF A HEALTHY ADULT PERFORMED ON TWELVE SUCCESSIVE DAYS

Day	1	2	3	4	5	6	7	8	9	10	11	12.	Mean	σ	V
Total red cells counted in 80 small squares	548	555	562	574	550	598	543	498	614	571	558	551	560	28	5 0%
Corresponding red-cell count (mill /c mm)	5 48	5 55	5 62	5 74	5 50	5 98	5 43	4 98	6 14	5 71	5·58	5 51	5 60		
Total red cells counted in 320 small squares	2,177	2,198	2,205	2,267	2,289	2,408	2,205	2,079	2,301	2,253	2,147	2,164	2,224	82	3·7%
Corresponding red-cell count (mill /c mm)	5 44	5 50	5 51	5 67	5 72	6 02	5 43	5 21	5 75	5 63	5 37	5·41	5 56		

The results of daily red-cell counts performed on a healthy adult Freely-flowing peripheral blood was obtained by ear prick and the same pipette and counting chamber used throughout. The cells were counted by the same (experienced) observer at his usual speed using a 4-mm objective and × 6 eyepieces The mean figures for the counts based on 80 or 320 small squares are almost identical The coefficient of variation has, however, been reduced, but not halved, by counting the larger number of cells The overall observed range of 5 21–6 02 million per c mm is produced by the summation of the " inherent " and " technical " errors of the counts as well as by a possible real day-to-day variation These figures give an idea of the fluctuations which may be expected in the blood count of a normal person They must be borne in mind when assessing the significance of changes in counts carried out on patients.

[illegible]

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millions per c.mm. (± 2σ), an enormous range! The coefficient of variation is increased in lower counts if the cells in 80 small squares only are counted. At the 3 million level it is 8·3% and at 2 million per c.mm. 9 0%.

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Corresponding red-cell count (mill./c.mm.)	5·44	5·50	5·51	5·67	5·72	6·02	5·43	5·21	5·75	5·63	5·37	5·41	5·56		

The results of daily red-cell counts performed on a healthy adult. Freely-flowing peripheral blood was obtained by ear prick and the same pipette and counting chamber used throughout. The cells were counted by the same (experienced) observer at his usual speed using a 4-mm. objective and × 6 eyepieces. The mean figures for the counts based on 80 or 320 small squares are almost identical. The coefficient of variation has, however, been reduced, but not halved, by counting the larger number of cells. The overall observed range of 5·21–6·02 million per c.mm. is produced by the summation of the "inherent" and "technical" errors of the counts as well as by a possible real day-to-day variation. These figures give an idea of the fluctuations which may be expected in the blood count of a normal person. They must be borne in mind when assessing the significance of changes in counts carried out on patients.

to some extent the final result. Another cause of error is due to selection of counting areas. There is a tendency to select areas whose cell content seems to fit in with a preconceived idea as to what the result should be. Undoubtedly, the best red-cell counts are made by an unhurried, honest, methodical worker who has no knowledge of what the result should be. He should always count the cells in the same areas in the counting chamber.

Further experiments on the selection of areas for red-cell counting have been carried out by Schneiderman, Mantel and Brecher (41). The experiments were designed to test, in counts based on the totals of cells in five groups of 16 small squares, whether the common practice of discarding the total for [illegible]

His results were obtained in the course of leucocyte counts, but probably the same principles apply to the distribution of red cells in the counting chamber. He recommends, therefore, that the centre of the cover-glass should exactly overlie the ruled area where the counts are performed

Alternative Methods of Red-Cell Counting

Photographic Methods

The errors of visual counting can be eliminated by taking photographs of the cells in the counting chamber after they have settled. The actual counts can be done at leisure later Technical details are given by Brown (8) and Swisher and Izzo (47)

Turbimetric Methods

Attempts have been made to count red cells (and measure their volume) [illegible]

corpuscular size and variation are a[illegible]
has been calculated that there is about a [illegible]
will show a difference of more than [illegible]
counts (of true value 5,000,000 per c mm.) where, in fact, no difference exists (38b).

Recently, however, the method has been improved, and an instrument [illegible]

Electronic Cell Counters

In recent years several different types of electronic equipment have been developed for the automatic counting of particles, and prototypes have been used to count red cells (30, 50). These machines have a very great potential advantage over the visual method of counting in that large numbers

is seldom great (see Chapter 5)

THE RETICULOCYTE COUNT

Reticulocytes are juvenile red cells; they contain remnants of the basophilic ribonucleoprotein which was present in larger amounts in the cytoplasm of the nucleated precursors from which they were derived. This basophilic material has the property of reacting with certain dyes such as brilliant cresyl blue to form a blue precipitate of granules or filaments This reaction takes place only in vitally-stained unfixed preparations If a blood film is allowed to dry and is afterwards fixed with methanol, the basophilic material gives rise to a diffuse basophilia when stained with basophilic dyes The most immature reticulocytes are those with the largest amount of precipitable material; in the least immature only a few dots or short strands are seen. Ripening with complete loss of basophilic material probably occurs as a rule in the blood stream after the cell has been delivered from the bone-marrow; the ripening process is thought to take 1 to 2 days—the exact time is uncertain.

As the number of reticulocytes in the peripheral blood is a fairly accurate reflection of erythropoietic activity, a reticulocyte count is one of the essential procedures of diagnostic haematology

A Technique for the Reticulocyte Count

The following technique gives reliable results

Diluting Fluid 1 0 g of brilliant cresyl blue (water-soluble, Gurr) dissolved in 100 ml of citrate-saline solution (one part 3% sodium citrate to four parts of 0·85% sodium chloride) The mixture is filtered after solution and is then ready for use

Method. 2 or 3 drops of the cresyl-blue solution are delivered by means of a Pasteur pipette into a small glass tube of 8 mm internal diameter. Two to four volumes of the patient's oxalated blood are then added, mixed with the cresyl-blue solution and the mixture placed at 37°C. in an incubator or water-bath for 15 to 20 minutes. At the end of this time the red cells are resuspended in the cresyl-blue solution

by gentle mixing and films made on glass slides in the usual way (see p. 37) When dry, the films are examined without being fixed or counterstained.

The exact volume of blood to be added to the cresyl-blue solution for optimal staining depends upon the red-cell count. A larger proportion of anaemic blood—and a smaller proportion of polycythaemic blood—should be added than of normal blood. In a successful preparation the reticular material should be stained deep blue and the *non-reticulated* cells shades of pale greenish blue.

Cresyl-blue stained films should not be counterstained, unless it is desired to keep them as permanent preparations. The reticular material is not better defined after counterstaining and precipitated stain overlying cells may cause confusion. Moreover, Heinz bodies will not be visible in fixed and counterstained preparations

Counting the Reticulocytes

An area of film should be chosen for the count where the cells are undistorted and where the staining is good. A common fault is to make the film too thin; however, the cells should not overlap. The cells should be counted using the 2-mm oil-immersion objective and if possible eyepieces provided with an adjustable diaphragm If eyepieces with an adjustable diaphragm are not available, a paper or cardboard diaphragm, in the centre of which a small square with sides about 4 mm in length has been cut, can be inserted into an eyepiece and used as a less convenient substitute

The counting procedure adopted should be appropriate to the number of reticulocytes present As can be seen in Table IV, very large numbers of cells have to be surveyed if it is desired to carry out reasonably accurate counts when only small numbers of reticulocytes are present

Haldane (16) showed that if amongst a population of cells a total of abnormal cells (in this case the reticulocytes) is counted during the course of examining a large number (N) of normal cells, the standard error can be derived from the formula $\sigma = p\sqrt{\frac{1-p}{m}}$, where p is the proportion of abnor-
... cells counted. Thus,
... al cells to be counted
... 01 (1%), to 72 when
... it is not necessary to
survey more than $240\left(\frac{72}{30} \cdot 100\right)$ *cells to obtain a standard error of 10%* when 30% of the cells are abnormal, if there are only 1% of abnormal cells, it is then necessary to survey as many as $10{,}100\left(\frac{101}{1} \cdot 100\right)$ cells in order to obtain a standard error of the same dimensions

The figures quoted above are obtained in the following way: if the desired standard error of the proportion of reticulocytes is 10%, then

$$\sigma = \frac{p}{10} \therefore \frac{p}{10} = p\sqrt{\frac{1-p}{m-2}} \text{ or } \frac{1}{10} = \sqrt{\frac{1-p}{m-2}} \text{ or } \frac{1}{100} = \frac{1-p}{m-2}$$

whence $m - 2 = 100 (1 - p)$ When $p = 0.01$, $m = 101$; when $p = 0.05$, $m = 97$; when $p = 0.3$, $m = 72$, and so on For practical purposes, $m = 100 - P$ (where P is the percentage of reticulocytes present) is a simple and sufficiently close approximation.

In Table IV is shown the total number of red cells that must be inspected to give approximately the same degree of accuracy of the reticulocyte count at different reticulocyte percentages The figures have been calculated from the above formulae.

TABLE IV

ACCURACY OF RETICULOCYTE COUNTS

Desired degree of accuracy (standard error (σ))	Percentage of Reticulocytes					
	1%	2%	5%	10%	25%	50%
20%	2,525	1,225	475	225	100	25
10%	10,100	4,900	1,900	900	400	100
5%	40,400	19,600	7,600	3,000	1,600	400

[illegible]

$P \pm 2 \times \frac{[illegible]}{100}$, e g, if P was 5%, then $P \pm 2\sigma = 5 \pm 2\%$ At least 475 cells would have to be counted to obtain this degree of accuracy

Fortunately, in practice it is not necessary to survey the very large totals of cells shown in Table IV when attempting accurate reticulocyte counts on blood containing only a small percentage of reticulocytes. As Woolf (53) and Brecher and Schneiderman (7) have pointed out, it is unnecessarily time-consuming to count very large numbers of *normal* cells In theory, for any degree of accuracy desired, there is no necessity to count a larger number of *normal* cells than abnormal cells—this is the principle of "balanced sampling" (53) Woolf (53) recommended surveying successive fields until the desired number of reticulocytes had been counted and counting the total cells (normal and abnormal) in sufficient fields—at least ten—to give a total number at least equal to that of the abnormal cells counted Brecher and Schneiderman (7) recommended the use of the Miller ocular, an eyepiece giving a square field in the corner of which is a second ruled square one-tenth the area of the large square They recommended counting

the reticulocytes in the large square (the whole field) and the normal cells in the small square and suggested that 20 fields should be counted as a routine—the magnification being so arranged that there were about 100 cells per field.

For counts less than 10% the author's practice is to survey successive fields until at least 100 reticulocytes have been counted (recording the number on a simple hand counter) and to count the total cells in every tenth field. The total cells in at least ten fields should be counted. The calculation is then simple:

Number of reticulocytes seen in 150 fields	=	100
Total cells present in 15 fields	=	300
∴ Approximate number of cells (all types) in 150 fields	=	3,000
∴ Reticulocyte percentage	=	$\frac{100}{3,000} \times 100$
	=	3·3%

When the reticulocyte count exceeds 10%, a greater number of complete fields will have to be counted in proportion to the fields in which only reticulocytes are counted As Table IV shows, only a relatively small number of cells will have to be surveyed to obtain a standard error of 10%.

It should be emphasized that it is essential that the reticulocyte preparation be well spread and well stained. Other important factors which affect the accuracy of the count are the visual acuity and patience of the observer and the quality and resolving power of the microscope. As in red-cell counts the most accurate counts are carried out by a conscientious technician who has no knowledge of the supposed reticulocyte level, thus eliminating the effect of conscious or unconscious bias.

The decision as to what is and what is not a reticulocyte may be difficult, as the most mature reticulocytes contain only a few dots or threads of reticular material. Nizet (37), who classified reticulocytes into groups according to the amount of basophilic material present,

containing "Pappenheimer bodies" (31)—generally only found in peripheral blood after splenectomy (see p 75) Fortunately, in well stained preparations, the Pappenheimer type of granular material—usually present as a single dot, less commonly as multiple dots—stains a darker shade of blue than does the filamentous material of the reticulocyte.

Normal Range of Reticulocyte Count

Adults .	0 2–2·0%
Infants (full-term, cord blood)	2–6%

ESTIMATION OF HAEMOGLOBIN

The large number of methods which have been suggested for the estimation of haemoglobin is evidence of the real difficulty that has attended this important estimatic [illegible] the preparation, calibration and [illegible] derivatives such as alkaline haematin and cyanhaematin keep relatively well and, although now outmoded, they have been quite widely used in techniques in which the haemoglobin sample to be tested is converted into the same derivative. Carboxyhaemoglobin and cyanmethaemoglobin are much more stable compounds and both derivatives have been extensively used in haemoglobin estimation—carboxyhaemoglobin for many years as the standard in Haldane's method, and cyanmethaemoglobin more recently (9, 12)

In the following section four procedures will be described and their merits and disadvantages discussed. Haldane's (carboxyhaemoglobin) method (15) is included for historical reasons rather than because it is recommended. The other methods to be described are the alkaline haematin method, an oxyhaemoglobin method employing either a visual reading photometer (M R.C. grey-wedge photometer (22)) or a photoelectric colorimeter, and the cyanmethaemoglobin method. There is little to choose in accuracy between the methods employing photoelectric colorimeters, although the alkaline haematin procedure is probably less accurate than the others Sahli's acid-haematin method appears to be less accurate than any of the methods mentioned, and will not be described, and the cyanhaematin method, although quite accurate, will also be omitted because it seems to have no particular advantage, and because it has the serious disadvantage of requiring the use of the poison sodium cyanide

The haemoglobin content of a solution may be estimated for standard or reference purposes by several methods; by measurement of its colour, its power of combining with oxygen or carbon monoxide, or by its iron content The clinical methods now to be described are all colour-matching techniques, which measure at the same time with varying efficiency any proportion of inert pigments (methaemoglobin or sulphaemoglobin) which may be present. Ideally, as a functional estimation of haemoglobin, measurement of oxygen capacity should be carried out, but this is hardly practicable in clinical practice It gives results at least 2% lower than the other methods (20) because a small proportion of inert pigment is probably always present. The iron content of haemoglobin can be estimated accurately but again the method is impracticable for clinical purposes. Estimations based on iron content are generally taken as authentic, but here, too, iron bound to inactive pigment is included. Iron content is converted into

haemoglobin content by assuming the following relationship: 100 g haemoglobin = 0·334 g. iron.

In practice it is far better for the accurate haemoglobin determinations required for the calibration of apparatus to be carried out centrally rather than for individual pathologists to attempt the rather difficult estimations required. In the Medical Research Council's scheme the haemoglobin content of the standard blood sample is estimated to within 1% by the use of three methods—iron determination, spectrophotometric measurement and a colour-matching procedure in a special comparator—and the blood sample* is dispatched in bottles containing a little streptomycin to act as antiseptic: in the American civilian and Armed Forces scheme the standardized sample is converted into a cyanmethaemoglobin solution (9, 12).

The attested whole-blood sample of the M R.C. scheme is intended to be used for the calibration of permanent standards, e g., Thomson's (48) grey solution,* neutral grey screens* (Ilford), Gibson and Harrison's (14) standard as used in the alkaline haematin method, or the carboxyhaemoglobin standard used in Haldane's method

Normal Range of Haemoglobin Content

Men		13·5–18 0 g. per 100 ml
Women		11 5–16 4 g „ „
Infants (full term, cord blood)		13 6–19·6 g. „ „
Infants (1 year)	(mean)	11 2 g. „ „
Children (10 years)	(mean)	12 9 g „ „

The Carboxyhaemoglobin Method (Haldane (15))

Method 0 02 ml of blood is washed into ammoniacal distilled water (0 04% (v/v) ammonia) previously placed in the graduated tube up to the 20 mark (The use of slightly alkaline water as a diluting fluid prevents the turbidity which often follows gassing. This is probably due to volatile acids such as sulphur dioxide which precipitate globulin) Coal gas is passed through the solution at a moderately brisk rate for at least 2 minutes by means of a glass capillary pipette. Frothing is prevented by first dipping the point of the pipette in caprylic alcohol The carboxyhaemoglobin solution is then carefully [illegible]

[illegible] normal [illegible] Haldane colour standard, [illegible] tion (B S I.), corresponds [illegible]

* Obtainable from Keeler Optical Products Ltd , 39 Wigmore Street, London W I

vith a haemoglobin content of 14 8 g per 100 ml by iron analysis and in oxygen capacity of 19·8 ml. per 100 ml (19a, 25)

The Haldane method can be made very much more accurate by comparing in a photoelectric colorimeter the intensities of light transmitted by the standard solution and by the test solution after carefully diluting the test solution exactly to the 100 mark (24)

The Oxyhaemoglobin Method

This is the most practical method for general use now that photoelectric colorimeters are widely available, the method is also suitable for use with visual photometers (see below)

(a) *Technique of the Photoelectric Method*

0 02 ml of blood is washed into 4 ml of 0 04% (v/v) ammonia contained in a tube provided with a tightly fitting rubber bung After mixing by inverting the tube several times, the solution of oxyhaemoglobin is ready for matching in the colorimeter A green filter (yellow-green, Ilford, No. 625) is employed If the optical density of the haemoglobin solution exceeds 0 7 the blood should be further diluted with an equal volume of distilled water

Standard At a dilution of 1 200, blood containing 14 g haemoglobin per 100 ml, placed in a 1-cm cell, gives an extinction coefficient of 0 475, using a yellow-green (Ilford 625) filter. A neutral grey screen of 0 475 density (Ilford or Chance) should, therefore, be used as a 100% standard With colorimeters fitted to take round test-tubes rather than rectangular cells, Thomson's solution (48), sealed in a test-tube, makes a convenient standard, and if diluted with one part of water to two parts of the solution, it also has an optical density of 0 475 (19)

However, colorimeters and light filters differ sufficiently one from the other to make it necessary to calibrate each standard in the colorimeter in which it is going to be used against blood of known haemoglobin content (19)

The advantage of the oxyhaemoglobin method is its simplicity and ness Its reliability is not affected by moderate grades of bilirubin

however, it is not satisfactory in the presence of carboxyhaemoglobin, methaemoglobin, or sulphaemoglobin. The artificial standards, although permanent, need accurate calibration.

As originally used, a disadvantage of the oxyhaemoglobin method was the tendency for the solution of oxyhaemoglobin to fade (46). This has been [illegible]

more at room temperature.

(b) Technique Using the M.R.C. Grey-Wedge Photometer (22).

The solution of oxyhaemoglobin is prepared as described above. The density of light transmitted by the test solution is matched in an adjacent half-field against the light transmitted by a rotating grey wedge. The same yellow-green filter (Ilford, No. 625) is employed as before. The haemoglobin content is read off as a percentage (Haldane scale, 100% = 14·8 g. Hb per 100 ml.).

With this instrument, the ability of oxyhaemoglobin to absorb green light is compared with a carefully calibrated neutral-grey wedge. The instrument is portable and may be used in daylight or artificial light. In careful hands it gives results comparable with photoelectric methods (21, 23, 29)

A possible cause of error is the presence of dirt on the walls of the glass cells or on the glass surfaces anywhere between the light source and the eyepiece, which affects the two light fields unequally. If the apparatus appears to be giving anomalous results, the first thing to do is to clean the glass surfaces thoroughly, taking it partially to pieces, if necessary

Sahli's method — [illegible]

Acid haematin [illegible]

The Alkaline-Haematin Method

The alkaline-haematin method is a useful ancillary method as it gives a true estimate of total haemoglobin even if carboxyhaemoglobin, methaemoglobin or sulphaemoglobin are present. A true solution is obtained, and the plasma proteins and lipoids have little effect on the development of colour, although they cause turbidity unless the blood and alkali are quickly and thoroughly mixed. A disadvantage of the method is that certain forms of haemoglobin are resistant to alkali denaturation, in particular the haemoglobin in foetal or neonatal blood (see p. 133), but this can be overcome by heating the solution in a boiling water-bath for 5 minutes. In normal circumstances, however, the method is more cumbersome and less accurate than the oxyhaemo- [illegible] photoelectric colorimeter.

[illegible] using [illegible] hod [illegible] l. of 0·1 N-NaOH and heated in a boiling water-bath for 3 minutes. It is

then cooled rapidly in cold water and when cool matched against the standard in a photoelectric colorimeter using a yellow-green (Ilford 625) filter.

Standard. This is a mixture of chromium potassium sulphate, cobaltous sulphate and potassium dichromate in aqueous solution (for preparation, see p. 220). The undiluted solution is equal in colour to a 1 in 100 dilution of blood containing 16·0 g. haemoglobin per 100 ml For use as a standard it should be diluted with distilled water so as to be equivalent to 7·4 g haemoglobin per 100 ml.

It is essential to heat the standard along with the test sample Only after heating, which alters the ionization of the salts it contains, does the ability of the standard to absorb green light approximate closely to that of alkaline haematin. A fresh sample of standard should be heated on each occasion and then discarded

(*b*) *Acid-Alkali Method.* A disadvantage of the alkaline haematin method as previously described is that the solution of haemoglobin in alkali has to be heated to ensure its complete denaturation This procedure can be omitted if the blood is collected first into acid and, after standing for 20 to 30 minutes, sufficient alkali is added to neutralize the acid and convert the acid haematin into alkaline haematin

0 05 ml of blood is washed into 4·0 ml. of 0 1 N-HCl and immediately well mixed with it. After standing for 20 to 30 minutes, 0 95 ml. of N-NaOH is added, and the tube is inverted several times. After standing for not less than 2 minutes, the test sample can then be matched in a photoelectric colorimeter A yellow-green filter (Ilford 625) is employed, using as standard a grey screen or solution (48) previously calibrated against blood of known haemoglobin content treated by acid and then alkali as described above

The Cyanmethaemoglobin Method (9, 12)

The basis of the method is to dilute blood in a solution containing sodium bicarbonate, potassium cyanide and potassium ferricyanide Haemoglobin, sulphaemoglobin, methaemoglobin and carboxyhaemoglobin are all rapidly converted to cyanmethaemoglobin The optical density of the solution is then measured in a photoelectric colorimeter, provided with a yellow-green filter (Ilford 625)

Method. 0 02 ml of blood is added to 5 ml of Drabkin's cyanide-ferricyanide solution ($NaHCO_3$ 1 g, KCN 0 200 g, $K_3Fe(CN)_6$ 0 200 g., distilled water to 1 litre) The tube containing the solution is stoppered with a rubber bung and inverted several times. After being allowed to stand at least 10 minutes at room temperature, the solution of [illegible] be compared with the sta[illegible]

[illegible] of known haemoglobin content diluted 1 in 250 in Drabkin's solution. Solutions equivalent to 5 g, 10 g and 15 g haemoglobin per 100 ml are convenient.

Solutions of cyanmethaemoglobin appear to last unaltered for years; they

pipettes or pipettes provided with a bulb

DETERMINATION OF PACKED CELL VOLUME (P.C.V. OR HAEMATOCRIT VALUE)

Haematocrit tubes are in daily use in most clinical laboratories, for the estimation of packed cell volume can be used as a simple and relatively accurate screening test for anaemia. In addition, in conjunction with accurate estimations of haemoglobin and red-cell count, knowledge of the packed cell volume enables the calculation of "*absolute values*" (see p. 54).

Wintrobe's Method

Wintrobe tubes, approximately 3 mm. in internal diameter and about 110 mm in height, calibrated at 1-mm. intervals to 100 mm, are usually employed for the determination of packed cell volume They hold about 1 ml of blood

Venous blood is collected with minimal stasis and rendered incoagulable by dry ammonium and potassium oxalate mixture, by Sequestrene or by heparin at concentrations of 2 mg per ml, 2 mg per ml. or 0·1 to 0·2 mg per ml, respectively

Potassium oxalate by itself causes the red cells to shrink and must not be used. The blood should be very carefully mixed by repeated inversion until bright red in colour, and the haematocrit tube then filled at once to the 100 mm mark by means of a glass capillary pipette. The tube is centrifuged in a centrifuge of 15 cm radius at a speed of 3,000 r.p.m. for 30 minutes—preferably for 60 minutes (see later) The height of the column of the red cells is taken as the packed cell volume (the volume occupied by the red cells expressed as a percentage of the total volume of the blood) Above the red cells and *not* included in the figure for the packed cell volume may be seen a greyish-red layer of leucocytes and above this, just below the plasma, a thin creamy layer of platelets

Normal Range for Packed Cell Volume (Haematocrit Value)

Men.	40–54%	Children (1 year) (mean)	35%
Women	36–47%	Children (10 years) . . (mean)	37·5%
Infants at birth (full-term, cord blood)			44–62%

...ly four times the amount of
...ven so, more than 1 litre of

ACCURACY OF THE HAEMATOCRIT METHOD

Duplicate samples centrifuged at the same time agree within 1%. It is this reproducibility which makes the estimation of packed cell volume so valuable. It is worthwhile to ensure that the blood is well aerated before filling the haematocrit tube as the packed cell volume of venous blood is appreciably (about 1%) greater than that of fully aerated blood (which has lost carbon dioxide and taken up oxygen). However, despite the ease and the close reproducibility of estimates of packed cell volume there are significant inaccuracies attached to the method as it is usually employed. Leaving aside inaccuracy due to irregularity of the bore of the tubes, failure to mix the sample of blood adequately or to incomplete filling or faulty reading, a further error of the method lies in the incompleteness of the packing of the red cells even though a constant volume is attained; indeed, the constant volume becomes, up to a point, progressively smaller as the speed of rotation is increased or, more correctly, as the centrifugal force to which the contents of the tube are exposed is increased (35).

In addition to the centrifugal force applied, the speed of packing depends upon the density and the size of the cells, the viscosity of the suspending fluid, and the relative densities of cells and fluid (38c).



fugal force of about 1,100 g (10).

The above considerations suggest that the conventional speed of centrifuging (3,000 r.p.m.) is too low or the maximum radius of the centrifuge (15 cm. as a rule) too small. The conventional method outlined above can

* The relation between relative centrifugal force (g), speed of rotation and radius of the centrifuge is given by the formula: Relative centrifugal force (R.C.F.) = $0.00001118 \times r \times N^2$, where r = radius and N = speed of rotation (r.p.m.).

and 1·5%, respectively, after 55 minutes' centrifugation Correction particularly

plasma trapping.

Micro-Haematocrit Methods

Several workers have used haematocrit tubes of much smaller diamet and capacity than that of Wintrobe tubes. The tubes are thus suitable f *haematocrit estimations on "capillary" blood (32, 42, 44)* Shils, Sass ar Goldwater (42) used 0 02-ml pipettes and found that values obtained wi

tube 32 mm in length, the centrifugal force was calculated to be abo 28,000 *g*. Packing was complete in 1 minute, and no correction for trappe plasma was thought to be necessary

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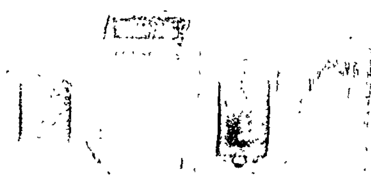


FIG 5 BLOOD FILMS MADE ON SLIDES

Left A well made film *Left centre* A film wh... irregular in thickness and which has b... A film which is too thick *Right* A film ... edged spreader and which shows long "



FIG 6 CENTRE (LEFT) AND TAIL (RIGHT) OF A BADLY MADE BLOOD FILM

The centre of the film is almost devoid of leucocytes, in the tail neutrophils, particularly, are present in large numbers 100

To face p 37

CHAPTER 3

BASIC HAEMATOLOGICAL TECHNIQUES: II

THE PREPARATION OF BLOOD FILMS ON SLIDES

BLOOD films can be made on slides or cover-glasses. The latter have the single possible advantage of a more even distribution of the leucocytes, but in every other respect slides are to be preferred. Unlike cover-glasses, slides are not easily broken; they are simple to label and when large numbers of films are dealt with slides will be found much easier to handle

Good films may be made in the following manner, using chemically cleaned slides wiped free from dust immediately before use —

A small drop of blood is placed in the centre line of a slide about 1 to 2 cm. from one end. The spreading slide is placed at an angle of about 45° to the slide and then moved back to make contact with the drop The drop should spread out quickly along the line of contact of the spreader with the slide The moment this occurs, the film should be spread by a rapid, smooth, forward movement of the spreader. The drop of blood should be of such a size that the film is about 3 cm. in length (Fig 5) It is essential that the slide used as a spreader should have an absolutely smooth edge and should be narrower in breadth than the slide on which the film is to be made, so that the edges of the film may be readily examined If the edge of the spreader is rough, films with ragged tails, containing many leucocytes, result (Figs. 5 and 6)

The faster a film is spread the more even and thicker will it be. A common mistake is to make films too thin whilst attempting to avoid the opposite (Fig 5) The ideal thickness is such that there is some overlap of red cells throughout much of the film's length with separation and lack of distortion towards the tail of the film The leucocytes should be easily recognizable throughout the length of the film Their distribution in films is discussed later (p. 42) (The preparation of films of aspirated bone-marrow is described on p 86)

Labelling Blood Films. A recommended method is to write the name of the patient and the date in pencil (graphite) on the film itself. It will not be removed by the staining A paper label may be affixed to the slide later

STAINING BLOOD AND BONE-MARROW FILMS

Romanowsky stains are almost universally employed for staining blood films, and very beautiful pictures may be obtained. For the best

results films should be stained as soon as they have dried in the air; they certainly should not be left for more than a few hours without fixation. If films are left unfixed for a day or more, it will be found that the background of dried plasma stains a pale blue and this is difficult to remove without spoiling the staining of the blood cells. As the intensity of staining is affected by any variation in the thickness of the film, it is not easy to obtain uniform staining throughout a film's length.

[illegible]

Giemsa's stain methylene blue azure is deliberately added to the methylene blue-eosin mixture. Jenner's stain is the simplest of the Romanowsky dyes now in use and Giemsa's stain the most complex Leishman's stain, which occupies an intermediate position is still widely used in the routine staining [illegible]

[illegible] eosin, and *vice versa*. A pH of 6 8 is recommended for general use, but to some extent this depends on personal taste A uniform pH is, however, [illegible]

[illegible] and its staining by eosin.

Staining Methods

Leishman's Stain

The film is dried in the air and the slide is then flooded with the stain After 2 minutes double the volume of distilled water is added [illegible] washed in a stream of [illegible] pinkish tinge (up to 2 minutes). After the back of the slide has been wiped clean, it is set upright to dry

Jenner-Giemsa Stain

The films are first dried in the air, then fixed by immersing in a jar of methanol for 10 to 20 minutes They are then transferred to a staining jar containing Jenner's stain freshly diluted with four parts of

buffered distilled water. After the films have been allowed to stain for approximately 4 minutes, they are transferred without washing to a jar containing Giemsa stain freshly diluted with nine parts of buffered distilled water After being stained for 7 to 10 minutes, the slides are transferred to a jar containing buffered distilled water, and rapidly washed in three or four changes of the water and finally allowed to stand undisturbed in the water for differentiation to take place. The time required varies; bone-marrow films often need from 3 to 6 minutes and peripheral-blood films somewhat longer (4 to 12 minutes) Differentiation may be controlled by inspection of the wet film under the low power of the microscope; with experience, however, the naked-eye [illegible]

No 1 cover-glass, using for this purpose a neutral mountant, such as Gurr's medium, which is miscible with xylol For a temporary mount, cedar-wood oil may be used

The cover-glass should be sufficiently large to overlie the whole film, including both the edges and the tail If a neutral mounting medium is used the staining should be preserved for at least 5 years Although it is probable that stained films keep best unmounted, there are objections to this course, it is almost impossible to keep the slides free from dust and in the absence of a cover-glass the observer is tempted to examine the film solely with the oil-immersion objective, a practice which is to be deprecated (p. 67)

The Jenner-Giemsa [illegible]

other without being allowed to dry. They may be allowed to dry after fixation, and staining can then be postponed for a few days However, in general, the sooner the films are stained the better the results

The diluted stains usually retain their staining powers sufficiently well for several batches of slides to be stained in them They must, however, be made up freshly each day, and it is probably best to stain the day's films all at the same time, if necessary in successive batches There is no need to filter the stains before use unless a deposit is present

May-Grunwald-Giemsa Stain

May-Grunwald stain may be substituted for Jenner's stain in the above-described technique Possibly better and brighter staining results The stain is used diluted with an equal part of buffered distilled water and the slides, after being fixed in methanol, are immersed in it for approximately 5 minutes before being transferred to the Giemsa

stain. They should be allowed to stain in the latter solution for 10 t[illegible] 15 minutes. Differentiation is carried out as described above.

Preparation of Solutions of the Romanowsky Dyes

Jenner's Stain 0 3 g of the powdered [illegible] to a c[illegible] added [illegible] room [illegible] for 24 hours, the solution is filtered. It is then ready for use, no ripening being required.

May-Grünwald Stain A 0 3% (w/v) solution is prepared in exactly the same way as [illegible]

[illegible]

FORMULAE OF PHOSPHATE BUFFERS

0 2 M-KH_2PO_4 (ml)	0·2 N-NaOH (ml.)	pH
50	17 8	6 6
50	21 0	6·7
50	23 7	6 8
50	26 5	6 9
50	29 6	7 0

LEUCOCYTE COUNTS

Total Leucocyte Counts

A 1 in 20 dilution of blood is made by adding 0 05 ml of blood to 0 95 ml of diluting fluid in a 75 × 8 mm. tube After tightly corking the tube, the suspension should be mixed by tilting and rotating by hand for at least 2 minutes or rotated in a cell-suspension mixer. The counting chamber is filled by means of a Pasteur pipette or stout glass capillary, as for red-cell counts (p. 12)

The red cells are haemolysed by the diluting fluid (see below) but the leucocytes remain intact, their nuclei staining deep violet-black. The preparation should be viewed with the 4-mm objective and × 6 eyepieces or the 16-mm. objective and × 10 eyepieces At least 100 cells should be counted in as many 1 sq. mm areas (0 1 c mm in volume) as may be necessary—the ruled area in a Neubauer chamber consists of nine of these areas

Calculation. If N cells are counted in 0 1 c mm , then the leucocyte count per c mm $= N \times 10 \times 20$ (dilution)
$= N \times 200$

Diluting Fluid 2% (v v) acetic acid coloured pale violet with gentian violet

Normal Range of the Total Leucocyte Count

Adults	4,000–10,000 per c.mm
Infants (full term, at birth)	10,000–25,000 ,, ,,
Infants (1 year) .. .	6,000–18,000 ,, ,,
Children (4 to 7 years)	6,000–15,000 ,, ,,
Children (8 to 12 years)	4,500–13,500 ,, ,,

THE ERROR OF THE TOTAL LEUCOCYTE COUNT

The factors causing errors in counting leucocytes are the same as in counting red cells As many leucocytes as possible should be counted, [illegible]

ponent of the error can be reduced to almost negligible proportions by using calibrated apparatus and avoiding " white-cell pipettes " which are difficult to manipulate

[illegible]

$\left(\frac{\sqrt{400}}{400} \times 100 \right)$

[illegible]

does not suggest that this is an important factor, for no significant difference was found between counts carried out on blood diluted in siliconed tubes, and in non-siliconed tubes, respectively Similarly, counts carried out on blood allowed to stand in the laboratory for 6 hours did not differ from [illegible]

several hours before undertaking the count

The Differential Leucocyte Count

The present discussion is concerned solely with the technique of performing differential leucocyte counts on blood films made on sl

For the purposes of a differential leucocyte count, the films must not be too thin and the tail of the film should be smooth, i.e., the films should be such that there is some overlap of the red cells, diminishing to separation near the tail, but it should not be so thick that the leucocytes in the body of the film are badly shrunken. If films are made too thinly, or if a rough-edged spreader is used, many of the leucocytes perhaps even 50% of them, become aggregated at the edges and in the tail (Fig. 6). Moreover, a qualitative irregularity in distribution is the rule; polymorphonuclear neutrophils and monocytes predominate at the margins and the tail, and lymphocytes in the middle of the film (Fig 7) This, perhaps, depends upon differences in stickiness, size and specific gravity among the different classes of cells. However, in well made films the departure from a random distribution is slight.

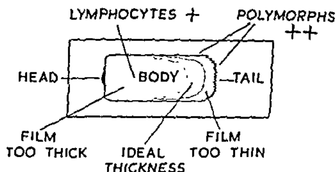


FIG 7 SCHEMATIC DRAWING OF A BLOOD FILM MADE ON A SLIDE
The film has been spread from left to right An indication is given of the way the *leucocytes are distributed (see text)*

Various systems of performing the differential count have been advocated. The problem is to overcome the differences in distribution of the various classes of cells, which are probably always present to a greater or lesser extent. *No system of counting* will compensate for the gross irregularities in distribution which occur in badly made films In films spread slowly by means of a spreader with an irregular edge the majority of the neutrophils will be carried to the tail of the film. There they will be seen in clumps and trails and many will be broken up and unrecognizable (Fig 6). *It is a waste of time to attempt a* differential count on such a film and, if this is attempted, futile to count only the cells in the centre of the film, where lymphocytes probably predominate, and to neglect altogether the tail, where the neutrophils lie. If the film has been well made, and many leucocytes are present in the body of the film and there is no great accumulation at the tail, the following technique of counting can be recommended:—

The cells should be counted, using the 4-mm. or the 2-mm lens, in a strip running the whole length of the film. The lateral edges of the

film are avoided. The film should be inspected from the head to the tail, and if less than 200 cells are encountered in a single narrow strip, one or more additional strips should be examined until at least 200 cells have been counted. Each longitudinal strip represents the blood drawn out from a small segment of the original drop of blood when it has spread out between the slide and spreader (Fig. 8). There is thus every reason to suppose that if all the cells are counted in such a strip, the differential totals will be representative of the true differential count. This technique admittedly does not allow for excess neutrophils and monocytes at the edges of the film, but this preponderance is slight in a well made film and in practice makes little difference to the result

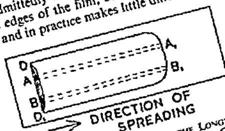


FIG 8 SCHEMATIC DRAWING ILLUSTRATING THE LONGITUDINAL METHOD OF PERFORMING DIFFERENTIAL LEUCOCYTE COUNTS

The original drop of blood spreads out between spreader and slide (D-D_1) The film is made in such a way that representative strips of films, such as A-A_1 and B-B_1, are formed from blood originally at A and B respectively In order to perform a differential count *all* the leucocytes in one or more strips such as A-A_1 and B-B_1 must be inspected and classified

The above technique is easy to carry out, with high counts (10,000 to 30,000 cells per c mm) a short film (2 to 3 cm) is desirable In patients with very high counts (as in leukaemia) the method has to be abandoned and the cells counted in any well-spread area where the cell types are easy to identify

A multiple manual register (22) is a help in recording the results of a count Most workers, however, find it possible to remember accurately the differential counts of small groups of 20 or 25 cells, writing the results on paper when each small group has been surveyed

Other systems of counting, such as the "battlement" count (15), seem to be more elaborate and have no advantage In the "battlement" count the cells in three longitudinal edge fields are first counted, then those in two transverse fields, then in two longitudinal fields, and then in two transverse fields back to the edge once more, and so on—three edge fields alternate with six internal fields This seems to weight the count too highly in respect of edge fields, for in a film 2 cm in width there may be as many as 50 fields across the width of the slide, when the 4-mm objective is employed

In Table V are given details of an experiment which de the reliability of the longitudinal-strip method of performing d counts The blood was derived from a normal subject. The are compared with a differential coun erformed on a "thick

(see p. 76) of the same blood As it is difficult to distinguish lymphocytes from monocytes in thick-film preparations they were classed together for the purposes of this experiment. Table V illustrates that the counts based on longitudinal strips gave almost exactly the same result as the counts based on the thick film. The table also gives an idea of the variation which may be expected among the counts on different strips if 200 to 300 cells are counted. It also reveals the increased numbers of leucocytes (and increased percentage of neutrophils) present at the margins of the films, and shows that counts performed on the head and body of the film give significantly lower neutrophil counts than are obtained when complete (head to tail) strips are counted, i.e., neutrophils are present in higher proportion at the tail.

TABLE V (a)

THE DIFFERENTIAL LEUCOCYTE COUNT

No	Total cells counted	Neutrophils Eosinophils Basophils	Lymphocytes Monocytes
1	100	71	29
2	100	61	39
3.	100	55	45
4	100	65	35
5.	100	69	31
6	100	72	28
7	100	69	31
8.	100	71	29
9.	100	73	27
10	100	65	35
	Total 1,000	Mean (a) 67%	Mean 33%

TABLE V (b)

No	Total cells counted	Neutrophils	Eosinophils	Basophils	%	Lymphocytes	Monocytes	%
11	300	186	0	0	62	98	16	38
12	225	157	3	1	71 5	61	3	28 5
13.	326	209	3	1	65 3	97	16	34 7
14.	327	224	3	4	70 7	86	10	29 3
15.	306	205	3	0	68	87	11	32
16	274	180	2	1	66 8	81	10	33 2
17	250	165	2	1	67 2	73	9	32 8
18	203	120	3	2	61 6	61	17	38 4
19.	328	224	3	1	69 5	86	14	30 5
20	300	198	6	1	68 4	91	4	31 6
	Total 2,840			Mean (b) 67 1% σ 3 2			Mean 32 9%	
21	417	298	2	3	72 7	87	27	27 3
22	398	288	5	2	74 1	81	22	25 9
	Total 815			Mean 73 4%			Mean 26 6%	

DIFFERENTIAL LEUCOCYTE COUNTS

TABLE V (c)

No	Total cells counted	Neutrophils	Eosinophils	Basophils	%	Lymphocytes	Monocytes	%
11A	200	114	0	0	57	74	12	43
12A	200	133	3	1	68·5	60	3	31·5
13A	200	115	2	0	58·5	71	12	41·5
14A	200	131	2	3	68	57	7	32
15A	200	120	1	0	60·5	72	7	39·5
16A	200	122	2	0	62	68	8	38
17A	200	112	0	4	61·5	71	6	38·5
18A	200	117	3	2	61·5	61	17	39
19A	200	125	2	0	62·5	64	9	37·5
20A	200	125	2	0	62·5	72	1	37·5
	Total 2,000				Mean (c) = 62·2% σ = 3·8			Mean = 37·8%
21A	200	141	1	0	72	42	16	28
22A	200	130	1	2	66·5	52	15	33·5
	Total 400				Mean 69·3%			30·7%

Differential leucocyte counts were carried out (*a*) on a thick-film preparation of normal blood (Table Va)—1,000 cells were counted in 10 groups of 100, and (*b*) on a thin film of the same blood made at the same time (Table Vb)—2,840 cells were counted from the head to the tail of the film in 10 internal longitudinal strips (11–20), and 815 cells in the two "edge" longitudinal strips (21, 22). In Table Vc (11A–22A) are recorded the differential totals for the first 200 cells encountered in each of the above longitudinal strips counting from the head towards the tail of the film. These totals were derived from the cells in about two-thirds to three-quarters of the length of the film. The mean granulocyte percentage (Mean (b), Table Vb) of the 10 complete internal longitudinal strips was 67·1%, and that of the incomplete longitudinal strips (Mean (c), Table Vc) was 62·2%. The difference between Mean (b) and Mean (c), 4·9%, is statistically significant. $t = 2·96$. $P = <0·01$ (for interpretation see text)

The variation among the counts made on different strips from the same film, as recorded in Table V, illustrates that the observed differential count depends not only on artificial differences in distribution due to the process of spreading, but also on "random" distribution, this latter effect is naturally reduced by counting a larger number of cells. Theoretical considerations and practical studies (11, 25) have shown that with a true neutrophil proportion of 50% the range ($\pm 2\sigma$) within which 95% of the counts will fall if 200 cells are counted is of the order of $\pm$ 7%, e g, 43% to 57% neutrophils, and if 100 cells are counted $\pm$ 10%, e g, 40% to 60% neutrophils. If 500 cells are counted the range would be reduced to 4·4%, e g, 45·6% to 54·4% neutrophils. With percentages above 50, the range is proportionally less, and below 50, greater than the above figures.

"Confidence curves" based on calculations of standard deviation, and assuming a perfect distribution, have been published (11). From these one can find the range within which lies the true value (95% probability) corresponding with any observed percentage count. The usefulness of these curves is limited because they can only be applied to well spread films. It is a waste of time to subject to statistical analysis results obtained from a badly film

Normal Range of Differential Leucocyte Counts in Adults

Neutrophils	.. ..	40–75%	2,500–7,500 per c.mm.
Lymphocytes	.. ..	20–45%	1,500–3,500 " "
Monocytes	.. ..	2–10%	200– 800 " "
Eosinophils	.. ..	1– 6%	40– 440 " "
Basophils	.. ..	< 1%	15– 100 " "

Differential Counts on Thick Films

The use of thick films for differential counts has already been referred to The disadvantage of the technique is that it is difficult to distinguish mononuclears from large lymphocytes; on the other hand, the method has the advantage that error due to an irregular distribution of the leucocytes is probably minimal, and since the leucocytes themselves are closely packed,

coated with silicone.

EOSINOPHIL COUNTS BY COUNTING-CHAMBER METHODS

Since the introduction of cortisone and A.C.T.H. into clinical medicine, the accurate counting of the eosinophils in the blood has assumed *some importance A number of different diluting* fluids have been proposed, and special counting chambers designed

The principles underlying the counting chamber or " wet " method of counting eosinophils have been well reviewed by Spiers (24) Ideally, the diluent should not only stain the eosinophil granules brightly and distinctly, but it should cause lysis of the red cells and of all other types of leucocytes

Diluting Fluids for Eosinophil Counts

The *acetone* group of diluents, introduced by Dunger (10), consist of. (*a*) an acid dye, such as eosin or phloxine, (*b*) distilled water to cause lysis of the red cells and rupture of the leucocyte membranes (the eosinophils seem more resistant than other leucocytes in this respect), and (*c*) acetone

eosinophil granules

Propylene glycol was first introduced as a diluent by Randolph (19, 20) The red cells gradually fade away in a dilution of 50% (v/v) propylene glycol, while the leucocytes remain uninjured. *The granules of the eosinophils can*

Method. 0 05 ml. (or 0·02 ml) of blood is added to 0·95 ml. (or 0 38 ml.) of diluting fluid so as to give a 1 in 20 dilution. After mixing the suspension for not longer than 30 seconds, the counting chamber is filled using a stout glass capillary or Pasteur pipette The eosinophils may be counted as soon as they have settled, or the count may be postponed for 30 minutes or so if the counting chamber is placed in a moist chamber (a Petri dish with cover containing a pledget of damp cotton wool)

Counting Chamber A chamber with the Fuchs-Rosenthal ruling, as used for cerebrospinal fluids, is suitable. Alternatively, a specially designed chamber with a larger ruled area may be used (16).

The ruled area in a Fuchs-Rosenthal chamber is a 4-mm square and the chamber is 0·2 mm in depth (area 16 sq mm and volume 3 2 c mm.). With counts at the upper limit of the normal range (see below) the whole ruled area should be surveyed and the total number of eosinophils recorded With lower counts, several fillings of the counting chamber must be surveyed It is convenient to use the 16-mm objective and × 10 eyepieces In a good clean preparation the eosinophils should be easily identified, their granules stain deep red and the cells containing them should be intact.

As in ordinary total leucocyte counts, the accuracy of the count is largely determined by the number of cells counted In a serious investigation 100 cells should be looked upon as the minimum, the counting chamber being filled several times, if necessary The coefficient of variation due to the random distribution of the cells is approximately ± 10% in a 100-cell count

Calculation. If N eosinophils are counted in 3·2 c mm., then the

$$\text{total eosinophil count per c mm} = \frac{N \times 20 \text{ (dilution)}}{3\ 2}$$

$$= N \times 6\ 25$$

Example If 100 cells are counted in the whole ruled area, total eosinophil count = 625 per c mm.

Diluting Fluid (10)

Eosin	0 1 g.
Acetone	10 ml
Distilled water	100 ml

Disintegration of the eosinophils takes place slowly in this diluting fluid and delay before the count is made is thus permissible However, it is probably best to fill the counting chamber as soon as the blood is diluted and to avoid prolonged mixing—in this way clumping of the eosinophils may be prevented (3)

Normal Range of Eosinophil Count

40 to 440 per c.mm (4, 17, 21).

at light (midnight) (5, counted the eosinophils their counts conformed

PLATELET COUNTS

Many methods for counting platelets have been published and th number is doubtless due to the real difficulty of counting small bodi which agglutinate and break up so easily, and which may be diffict to distinguish from extraneous matter. No attempt will be made review recommended methods in detail; the reader is referred to tl excellent reviews of Tocantins (27, 28) for technical and other detai concerning mammalian platelets. In the present chapter two simp techniques only will be described: Lempert's modification of Kriste son's method (13) and a method employing the formal-citrate dilutir fluid used in red-cell counts Both methods are suitable for use wit venous blood; Lempert's method can also be used with capillary bloo

Lempert's Modification of Kristenson's Method (13)

Two solutions are required·

Solution *A*	Sodium citrate	1 0 g
	Mercuric chloride	0·002 g.
	Brilliant cresyl blue	*0 2 g*

The reagents are dissolved in 100 ml of distilled water warmed tc 45°C.

Solution *B*	Urea	20·0 g
	Distilled water	*to 100 ml*

Equal volumes of solutions *A* and *B* are mixed when required The mixture will keep for several days, but must be filtered or centrifuged before use

The patient's ear is warmed by rubbing with lint The mixed stain is drawn up in a leucocyte-counting bulb pipette to the 0 5 mark The patient's ear is pricked sufficiently deeply to obtain a free flow of blood Blood is taken up into the pipette so that the upper level of the diluting fluid reaches the 1 0 mark, and then the pipette is filled with stain up to the 11 mark The pipette is shaken for 1 minute. The contents of the pipette are remixed for 2 minutes before a Neubauer counting chamber is filled This is placed in a Petri dish containing a pledget of damp cotton wool and allowed to stand for 20 minutes to 1 hour, to ensure sedimentation of the platelets and lysis of the red cells.

The platelets are counted under the 4-mm objective The red cells

Calculation. If N be the number of platelets counted (in 80 small squares), the number in 1 c.mm. $= \frac{N}{80} \times 4{,}000 \times 20$ (dilution)

$$= N \times 1{,}000.$$

Lempert's method is fairly satisfactory. It has the advantage that the platelets are counted directly. They do not seem to agglutinate, but they are slow to settle in the counting chamber and may be difficult to see. The red cells sometimes haemolyse slowly, and it is usually best to leave the preparation in the moist chamber for an hour before counting the platelets The method is unreliable in the presence of many reticulocytes, for basophilic material which may be confused with platelets is liberated when the reticulocytes are haemolysed

Other bodies which may be mistaken for platelets include fragments of leucocyte cytoplasm—sometimes seen in leukaemia, particularly the monocytic type, and " Pappenheimer bodies " (see p 75) Cytoplasmic fragments [illegible]

It is essential that the pipette and counting chamber be absolutely clean and that the staining solution be filtered immediately before use The urea solution appears to lose its haemolytic power on keeping and should be renewed every week or so

A Method Using a Formol-Citrate Diluent

In the method now to be described venous blood is added to the diluting fluid in a proportion of 1 to 100, and the number of platelets counted in a counting chamber

Diluting Fluid 1% (v/v) formalin in 3% trisodium citrate solution. One or two drops of 1% brilliant cresyl blue in isotonic saline may be added to each litre of formol-citrate solution, but this is a matter of personal choice In any event the solution must be filtered immediately before use 10 ml are then delivered into a clean 1-oz. screw-capped bottle.

Method Blood is withdrawn after a clean venepuncture into a dry syringe using a short needle of 19 or 20 S W G bore The needle is then detached from the syringe and a small volume of blood is delivered on to a watch-glass whose surface has been covered with paraffin wax This blood is immediately diluted 1 in 100 in the formol-citrate solution A straight 0 2-ml pipette calibrated at 0 1 and 0·2 ml is used to make the dilution, the diluting fluid is taken up to the 0 1-ml. mark and 0 1 ml of blood is then taken into the pipette so that the upper level of the diluting fluid reaches the 0 2-ml mark The contents of the pipette are washed into the 9 9 ml of diluting fluid remaining in the screw-capped bottle The suspension of blood is immediately well mixed by inverting the bottle several times

The contents of the bottle are again mixed for at least 2 minutes before a Neubauer counting chamber is filled with the suspension,

using a stout glass capillary or Pasteur pipette. The counting chamber is placed in a moist chamber and left untouched for at least 10 minutes to give time for the platelets to settle.

The preparation is examined with the 4-mm. objective and × 6 or × *10* eyepieces. The platelets appear under ordinary illumination as small (but not minute) highly refractile particles, if viewed with the condenser racked down; they are usually well separated (for exceptions to this, see later) and stain a very pale blue when viewed with a bright light, if dye has been added to the diluting fluid. The platelets are more easily seen with the phase-contrast microscope (7), but this apparatus is by no means essential.

The number of platelets in one or more areas of 1 sq. mm. should be counted The total platelets counted should always exceed 100—this number present in an area of 1 sq. mm. corresponds with a count of 100,000 per c mm.

Calculation. If N be the number of platelets counted in an area of 1 sq. mm. (0·1 c.mm in volume), the number of platelets per c.mm.
= N × 10 × 100 (dilution)
= N × 1,000

The above simple method is satisfactory in practice. As a rule the platelets are well separated and clumps are few. However, with high counts, such as may be found after splenectomy, clumps may form Collecting the blood in a siliconed syringe may help to prevent clumping. *Usually, however, it is unnecessary to use a siliconed syringe.* The platelets disintegrate only slowly in the diluting fluid and the suspensions once diluted may as a rule be left for several hours without harm before the counts are made.

The technique described above is a practical one, for venous blood collected without special precaution can be used provided the dilution for the platelet count is made as soon as the blood is withdrawn. As platelets clump rapidly in oxalated blood, it is useless to attempt to *count platelets in oxalated blood collected in the ward and subsequently* submitted to the laboratory It is, however, possible to do this if Sequestrene is used as an anticoagulant Indeed, Sequestrene has such a remarkable property of preventing platelet clumping that perhaps the *best method of dealing with blood containing rapidly clumping* platelets (as after splenectomy) is to collect the blood with a siliconed syringe and to add it to Sequestrene before making the dilution for the platelet count. It should be emphasized that, *if difficulty is experienced in withdrawing blood from a vein,* it is useless to try to count the platelets in it A clean venepuncture is essential.

Normal Range of Platelet Count

The results obtained by the Lempert-Kristenson method and the formol-citrate method are essentially similar. The normal range in

health by both methods is approximately 150,000 to 400,000 per c.mm No sex differences have been detected and the counts in individual subjects are relatively constant (7, 23). According to Sloan (23) diurnal variation is not significantly greater than the error inherent in the counts

It is a matter of personal preference which of the two methods is used. In either case experience and practice is required before much reliance can be placed upon the results. In the Lempert-Kristenson method, although large numbers of platelets are available for counting, they may be difficult to see and to distinguish from extraneous particles In the formol-citrate method, though the platelets are easier to see in a good preparation, relatively few can be counted within a reasonable time and this and the increased likelihood of clumping are additional sources of error. Fortunately, the general trend of the platelet count is all that is usually required for clinical purposes. Certainly, no significance at all should be placed upon differences in platelet counts of less than 25%. Accuracy in counting is unlikely to be achieved except by the most careful regard to detail and only in the hands of an experienced worker. The error in platelet counting is considered by Biggs and MacMillan (6), Brecher and Cronkite (7), Sloan (23), and Brecher, Schneiderman and Cronkite (8) In careful hands, however, the error should not exceed that of other chamber-counting methods, and as in red-cell counts the most important single factor in reducing the error of the count is to count as many cells as possible.

The "normal" range for platelet counts in the literature is astonishingly large, being from 150,000 to 600,000 per c mm. or more (23) Although there is a considerable real variation from person to person, much of this wide range is due to differences in technique The highest values are obtained by the use of the 2-mm oil-immersion objective, but there is no reason to suppose that high counts are necessarily correct ones, probably fragments of platelets or other particles are included in the count Venous blood appears to contain more platelets than does capillary blood, and methods employing venous blood are to be preferred Probably some platelets always adhere to the edges of a skin puncture wound, and are thereby lost In practice, the results with peripheral blood are less constant than with venous blood (8)

SIGNIFICANCE OF THE PLATELET COUNT

As referred to later, a platelet count should be carried out on every patient who presents as a haematological problem, for the result is often of real importance in diagnosis A persistently reduced count (<100,000 per c mm) is a pointer to a definite abnormality affecting haemopoiesis or the haemopoietic tissues which may range from aplastic anaemia and leukaemia to thrombocytopenic purpura and carcinomatosis A platelet count persistently above the normal range (>500,000 per c mm) is less frequently found, but here, too, the observation is often of diagnostic importance, as for instance in polycythaemia vera

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CHAPTER 4

MEASUREMENT AND CALCULATION OF SIZE OF RED CELLS

"ABSOLUTE VALUES"

THE mean cell (corpuscular) volume (M.C.V.), mean cell (corpuscular haemoglobin (M.C H.) and mean cell (corpuscular) haemoglobin concentration (M.C.H C.) are generally referred to as "absolute values". These values, calculated from the results of the red-cell count, haemoglobin concentration and packed cell volume, have been widely used in the classification of anaemia, and their introduction has rendered obsolete the various indices, such as the "colour index" which were derived from comparison of the observed data with figures arbitrarily assumed to be normal

However, although knowledge of the absolute values in an anaemic patient may be of definite scientific value, their calculation often fails to contribute decisively to an accurate diagnosis (see Chapter 5). This is because macrocytosis or microcytosis, for instance, are not changes specific to any particular type of anaemia and also because it is in any case extremely difficult to make accurate measurements Nevertheless, it has to be admitted that the focusing of attention on the variations in mean cell volumes and mean cell haemoglobin content and concentration in health and disease, and also the measurement of red-cell diameters by Price-Jones, have made a significant contribution to the understanding and classification of the anaemias, even if classifications based simply on cell dimensions are now becoming outmoded.

Calculation of Mean Cell Volume (M.C.V.)

If the packed cell volume and the number of red cells per c.mm are known, the mean cell volume can be calculated:

e g., if the packed cell volume is 45%, in 1 c mm. of blood there are 0 45 c mm of red cells

∴ If there are 5,000,000 red cells per c mm, they occupy a volume of 0·45 c mm.

$$\therefore \text{Volume of 1 cell} = \frac{0\cdot45 \text{ c.mm}}{5{,}000{,}000} = \frac{0\cdot45 \times 10^9}{5 \times 10^6} \text{ c}\mu.$$

$$= \frac{0\cdot45 \times 10^3}{5} = 90 \text{ c.}\mu.$$

In practice, the packed cell volume as a percentage is divided by the

red-cell count in millions per c mm, and multiplied by 10 The answer is expressed in cubic micra.

e.g, $(45 \div 5) \times 10 = 90$ c.μ

Calculation of Mean Cell Haemoglobin (M.C.H.)

This can be calculated if the haemoglobin and red-cell count are known.

e.g, if there are 15 g haemoglobin per 100 ml blood, there is $\frac{15}{100 \times 1{,}000}$ g Hb per 1 c mm. blood

. If there are 5,000,000 red cells per c mm, the mean cell haemoglobin is $\frac{15}{100 \times 1{,}000 \times 5{,}000{,}000}$ g per cell

$= \frac{3}{10^{11}}$ g

= 30 micromicrograms (μμg)

Calculation of Mean Cell Haemoglobin Concentration (M.C.H.C.)

This can be calculated if the haemoglobin concentration per 100 blood and packed cell volume are known

e.g, if there are 15 g haemoglobin per 100 ml blood, of pack cell volume 45%, the mean cell haemoglobin concentration $15 \div 45$ g. % = 33 3%

Range of "Absolute Values"

Normal (10, 13)

Mean cell volume (M C V) 76-96 c μ

Mean cell haemoglobin (M C H) 27-32 μμg

Mean cell haemoglobin concentration (M.C.H.C) 32-36%

In Disease

(a) In the macrocytic anaemias

M C V. increased up to about 150 c μ (rarely higher),

M C H increased up to about 50 μμg (rarely higher),

M C H C normal or diminished

(b) In microcytic hypochromic anaemias

M C V diminished to 50 c μ (rarely lower),

M C H diminished to 15 μμg (rarely lower),

M C H C diminished to 22% (rarely lower)

THE ACCURACY OF THE CALCULATION OF "ABSOLUTE VALUES"

A danger attached to the calculation of absolute values is that the observer may delude himself into a false sense of their accuracy, particularly when the results are expressed to one place of decimals, as is sometimes quite unjustifiably done.

The significance and accuracy of absolute values naturally depends upon the accuracy of the estimations from which they have been derived. As has already been mentioned, the error of the estimation of packed cell volume can be reduced to small dimensions, not more than ± 1%, and the same is more or less true of haemoglobin estimation, which in the very best hands has a minimum error of about ± 2%. Thus it is possible to obtain a reasonably good estimate of mean cell haemoglobin concentration. Unfortunately, the same cannot be said of the calculation of mean cell volume or mean cell haemoglobin. These estimations depend for their accuracy largely on the accuracy of the red-cell count. Now, although it is possible to reduce the error of the count to small proportions, even to as little as ± 2%, this can only be done by counting thousands of cells, which can rarely be carried out in practice. If the red-cell count is done in a "routine" manner—with perhaps not more than 500 cells counted—the potential error is so large that any calculation of mean cell volume is almost worthless. An example will make this clear. The error of a red-cell count comprising a count of 500 cells in 80 small squares cannot be less than ± 8·8% (see p. 17). Thus blood of real red-cell count 5·0 million per c.mm. might appear to contain anything between 4·56 to 5·44 million red cells per c.mm. (± 2 σ limits). If the packed cell volume of the same blood was 47%, the mean cell volume would appear to be within the range 103 c μ. to 86 c μ., with a 1 in 20 chance of being outside this range. Hence the above-mentioned blood might or might not seem to be abnormal, depending on chance selection of the areas surveyed in the counting chamber In fact, the average of repeated observations on the same blood, or a single count based on several thousand cells, is necessary to determine reliably slight deviations from the normal.

MEASUREMENT OF RED-CELL DIAMETERS

Normally, even in health, there is a readily appreciable variation in diameter between a population of red cells It was largely owing to the work of Price-Jones (9) that this variation was placed on a quantitative basis He showed that if the diameters of a large number of red cells were measured and the cells grouped together in classes according to their diameters, the frequency-distribution curve of diameters was of the "normal" type Price-Jones applied statistical methods to this data and worked out the limits of normal variation in great detail; he also demonstrated that characteristic deviations from the normal were encountered in various types of anaemia. This work excited great interest, and at one time the drawing of a Price-Jones curve was deemed an almost essential step in the investigation of any obscure case of anaemia—although the labour expended contributed nothing to the understanding of the case and merely placed on paper what was to be seen by inspection of a stained film!

nor shrunken is therefore required before measurements are undertaken. Moreover, in an anaemia such as severe pernicious anaemia it is extremely difficult, if not impossible, to measure accurately the many abnormally shaped

distribution curves to be obtained as a rule. The accuracy of the estimation of the mean cell diameter is hardly affected by increasing the number of cells counted from 100 to 500, the standard deviation, however, will be reduced by counting the larger number of cells

There exist at least two other possible methods for the measurement of red-cell diameters in addition to Price-Jones's original technique and later variants of it. these are measurements by means of a micrometer scale introduced into the microscope eyepiece and the use of the diffraction (halometric) method, based on an entirely different principle. Brief descriptions of all three techniques are given below

THE PRICE-JONES TECHNIQUE

The basic principle of this technique is the outlining with a sharp pencil of the individual images of red cells projected on to a white screen at a magnification of × 1,000 The outlines are measured later by means of a millimetre rule

In order that the cells be clearly seen, the film should be deeply stained with eosin after fixation in methanol It is convenient to place the microscope horizontally and by means of a prism attached to the eyepiece or by means

lower power objectives the focus of the microscope is adjusted so that the micrometer scale is projected sharply on to the piece of paper in the centre of the field The 2-mm. oil-immersion objective is next used and the image

periphery of the fields, which are not in sharp focus.

When the outlining of the cells has been completed, in perhaps an hour, the diameters of each cell are measured using a glass millimetre scale which

can be closely applied to the outlined cells The greatest and smallest diameters of each are measured to 0 5 mm, the mean of these two measurements being accepted as the diameter of the cell. The calculated diameter should be written within the drawing of each cell, as soon as it has been measured.

When the measurements have been completed the diameters are grouped in intervals of 0 25 μ The totals (frequencies) of each class-interval may be plotted on graph paper as a grouped frequency-distribution curve.

Various modifications of Price-Jones's technique have been proposed in order to save time and to avoid the errors introduced by first outlining the cells and then measuring the outline. It is possible to measure directly the images of the projected cells with a glass or plastic rule and to write directly on to the cells' images on the paper the mean of two measurements at right angles. In this way the diameters of 500 cells may be measured and scored within an hour At a magnification of 2,000 times a measurement of 15 mm corresponds to 7·5 μ. Alternatively, the diameter may be measured by finding the best fit with a series of black rings of different sizes drawn on to the paper on which the images of the cells are projected (5). Another modification is that of Hynes and Martin (6) who, using the microscope in a vertical position, project the film on to a ground glass screen at a magnification of × 2,000. The diameters of the cell images are measured by means of a celluloid protractor on which circles of a range of diameters are drawn Finally, it is possible to photograph the projected images of the cells and to measure them at leisure (1).

Statistical Method for Dealing with Measurements of Red-Cell Diameters (after Price-Jones (9))

The accompanying table (Table VI) is based on Price-Jones's procedure and shows how it is possible to calculate the arithmetic mean (M), the standard deviation (σ) and the coefficient of variation (V) of the *diameters of the red cells of a sample of blood*

Arithmetic Mean (M) The class-intervals are tabulated in column 1 and corresponding frequencies of diameters in column 2 In order to obtain [illegible]

middle of the range (in the particular case given in the table, 6 75 μ [illegible] then to mark off in colum[illegible]

[illegible]

The sum (−50) is then [illegible] the real and arbitrary mea[illegible] units The true mean is thus 6 75 μ [illegible] 6 72 μ

Standard Deviation (σ) The standard deviation is a measure of the

TABLE VI

STATISTICAL METHOD FOR DEALING WITH MEASUREMENTS OF RED CELL DIAMETERS
[After Price-Jones (9)]

1 Mid-point of class intervals (in 0·25μ units)	2 Frequencies	3 Deviation from arbitrary mean (6·75μ) (class interval units)	4 Product (2 × 3)	5 Product (2 × 3²)
5·5	10	−5	50	250
5·75	18	−4	72	288
6·0	42	−3	126	378
6·25	70	−2	140	280
6·5	80	−1	80	80
			−468	
6·75 (arbitrary mean = A)	83	0		
7·0	82	+1	82	82
7·25	51	+2	102	204
7·5	33	+3	99	297
7·75	20	+4	80	320
8·0	11	+5	55	275
			+418	2,454
			468	500 = 4·91
			−50	

$$M \text{ (true mean)} \quad A \text{ (arbitrary mean)} \quad \frac{-50}{500} (0.25\mu)$$
$$= -0.1 (0.25\mu)$$
$$= -0.025\mu$$
$$M = 6.75 - 0.025\mu$$
$$= 6.72\mu$$

$$\sigma^2 = 4.91 \quad (M - A)^2 (0.25\mu)$$
$$= 4.91 \quad 0.01 (0.25\mu)$$
$$= 4.9 (0.25\mu)$$
$$\sigma = 2.2 (0.25\mu)$$
$$= 0.55\mu$$

$$V = \frac{\sigma}{M} \times 100$$
$$= \frac{0.55 \times 100}{6.72}$$
$$= 8.2\%$$

[illegible]

scatter or dispersion of the diameters, both their range in size and their frequency distribution. The scatter about the mean implies diameters less (−) and diameters greater (+) than the mean diameter. In order to obtain some quantity that varies with the overall dispersion, it is necessary to average the deviations by a process that treats them as if they were all of the same sign, and squaring is the simplest process for this purpose.

Column 5 (*Table VI*) gives values for the squared deviations (column 3 multiplied by column 4). Addition of these products gives 2,454 which divided by 50[illegible] trary mean (A [illegible] This subtract[illegible] thus 2·2 in 0 [illegible]

Coefficient of Variation (V). The coefficient of variation measures the degree of scatter of the distribution in relation to the mean value of the item varying. It is the standard deviation (σ) expressed as a percentage of the mean: i.e., $V = \frac{\sigma \times 100}{M}$ It is a measure of variability which is independent of the unit in which the measurements have been made

In the example given, $V = \frac{0.55 \times 100}{6.72} = 8.2\%$

The diameter distribution curve based on the above data is illustrated in Fig. 9 (right-hand curve)

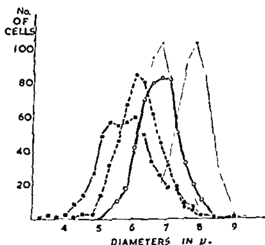


FIG. 9 RED-CELL DIAMETER DISTRIBUTION CURVES (PRICE-JONES CURVES)

o——o is a curve constructed from the blood of a mild case of hereditary spherocytosis—see note added to legend to Table VI ● - - - ● and ■ ═══ ■ are curves drawn from the blood of two patients suffering from typical hereditary spherocytosis The characteristic microcytosis is well demonstrated. The thin lines indicate the maximum and minimum normal curves (data from Price-Jones (9))

Normal Range for Red-Cell Diameter (Dry Films) (Price-Jones (9))

Mean cell diameter (M C D)	6 7–7 7 μ (mean 7·2 μ)
Standard deviation (σ)	0 35–0·56 μ
Coefficient of variation (V)	5 3–7 3% (mean 6·3%)

Measurement of Red-Cell Diameters by the Diffraction Method

The diffraction method for measuring the size of small objects was first applied to the measurement of the diameters of red cells by Pijper in 1919. The principle of the method is that when a parallel beam of polychromatic light is passed through a film of relatively opaque red cells the light is diffracted by each individual red cell, the degree of diffraction varying with the cell diameter The individual spectra appear to the human eye as a concentric compound spectrum surrounding the source of light, it may be projected as an image on a screen by means of a convex lens.

Each diffraction pattern consists of a bright centre, surrounded by an orange ring, and then the spectral colours in their natural sequence from violet to red. Outside the red ring fainter green and red circles may be seen

The size of the concentric spectral rings varies inversely with the diameters of the red cells and directly with the distance of the film from the light source In macrocytic anaemias the spectral circles are smaller than normal and in microcytic anaemias they are larger The distance between the violet and red circles gives an expression of the degree of anisocytosis. As the spectral rings are produced by the blending of rings of different sizes depending upon the varying diameters of the red cells, the smaller the degree of anisocytosis the narrower and clearer are the bands; conversely, if there is much variation in cell size, the spectral pattern is much less clear

The diffraction method of measuring red-cell diameters has been widely used in clinical haematology, and though the method is hardly precise, it has the great advantage of being extremely quick A well spread film is required, and in the area examined the red cells should be undistorted and just not overlapping If the film is too thin, the spectra are weak in intensity It is best to examine an unstained film

[illegible]

volume by means of the diffraction technique The results agree closely [illegible]

appears as a black ring. This is viewed through an eyepiece into which is inserted a micrometer scale calibrated to 0·1 mm In this way the diameter of the black ring can be measured and the corresponding mean cell diameter calculated from a formula.

Another method suggested is that of Emmons (3) [illegible]

[illegible] the film and the source of light [illegible]

[illegible] use of an eyepiece micrometer are given in the papers of Haden (4) and Pijper (7). Haden found that Emmons's eriometer and Pijper's blood-cell tester gave the best results

The diffraction method is certainly a useful way of estimating red-cell diameters approximately, and the experienced observer will be able [illegible] alteration in mean cell diameter but also depend- [illegible] so- [illegible] the information obtained by the use of diffraction methods is often of decisive use in clinical haematology It is true that the method may sometimes call attention to unexpected macrocytosis. This is, however, likely to be equally obvious to the experienced eye—aided perhaps by an eyepiece micrometer—when the film is viewed with the microscope. Certainly, the diffraction method of measuring diameters is no substitute for microscopy, which reveals so much besides.

Measurement of Red-Cell Diameters with the Aid of an Eyepiece Micrometer

The eyepiece micrometer scale can be usefully employed in the measurement of cell diameters It has the advantage of directness and simplicity and can be quickly applied However, when a relatively large number of cells are to be measured it is simpler and quicker to project them at a magnification of × 1,000 or × 2,000 and to measure the images by one of the methods described earlier in this chapter

The scale of the eyepiece micrometer has to be calibrated in relation to the objective, eyepiece and tube-length employed, before it can be used This is best done using a slide on which a scale, usually 1 mm. in 0·01-mm (10 μ.) intervals, has been engraved Alternatively, the calibrations on a counting chamber can be utilized (the side of a smallest square is 0 05 mm. in length).

It is convenient to have a conversion scale kept near the microscope; e.g., using a 2-mm. objective and × 6 eyepieces

5·0 divisions = 6·6 μ
5·5 " = 7 2 μ.
6·0 " = 7 9 μ.
6 5 " = 8 5 μ., etc

The diameters of red cells can be measured to about 0 5 μ. without difficulty with the aid of the eyepiece micrometer Although minor degrees of deviation from the normal will not be detected, the method is useful, and in practice it is possible by measuring a few cells whose diameters are representative to confirm or refute visual impressions of abnormalities in cell size. This does not mean that the observer should search for the largest or smallest cells to measure, a few as large as 9 μ or as small as 6 μ. may be found in normal blood It is much more significant to find an unusually high proportion of cells of 8·5 μ than a few outside these limits. Price-Jones's (9) ranges in health (± 3σ) give the outside normal limits for cells of the dimensions just referred to.

CALCULATION OF MEAN CELL THICKNESS (M.C.T.)

Mean cell thickness can be calculated from knowledge of the mean cell diameter and mean cell volume, assuming the red cell to be a short cylinder rather than a biconcave disc On this assumption M C T (or more correctly M C.A.T. (mean cell average thickness))

$$\frac{M\ C\ V}{\pi\left(\frac{M\ C\ D}{2}\right)^2}$$

The normal range is from 1 7 to 2 5 μ

It is obvious that a figure for M C T, derived as shown above, can at the best be looked upon only as an approximate measurement, and it is doubtful whether its calculation has any practical value

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CHAPTER 5

USE OF BASIC HAEMATOLOGICAL TECHNIQUES IN CLINICAL MEDICINE

LITTLE has been said in the foregoing chapters about the way in which the simple techniques that have been described should be used in clinical work. The problem presents in three ways: (*a*) the differentiation of abnormal from normal blood findings; (*b*) the diagnosis of a particular blood abnormality or disease, and (*c*) the haematological investigation of a patient who is to be studied in detail. Each type of problem needs a different approach.

DIFFERENTIATION OF ABNORMAL FROM NORMAL BLOOD FINDINGS

As the blood findings of the great majority of patients investigated are likely to be normal, a simple and time-saving technique is required. In most instances it is sufficient to estimate the haemoglobin content of capillary blood and to examine a stained blood film These procedures constitute the minimum possible "screening" test. Determination of the packed cell volume can be used instead of haemoglobin estimation if venous blood is available A red-cell count, however, is entirely out of place as a screening procedure, for it is impossible, by visual methods of counting, to perform an accurate count quickly

Blood for a leucocyte count should be taken at the same time as that for haemoglobin estimation, but if the leucocytes appear normal in the stained film the total count need not be done, unless there is some special indication for a study of the leucocytes, e g , in infections when even a normal total count may be of significance. Even if the haemoglobin content or packed cell volume of the blood is normal, a film should be made and stained Although there may be no anaemia, inspection of the film may show the presence of unsuspected blood diseases, e g, leukaemia (particularly chronic lymphatic leukaemia), mild megaloblastic anaemia, compensated haemolytic anaemia or congenital abnormalities such as elliptocytosis

DIAGNOSIS OF THE TYPE OF BLOOD DISORDER

For most clinical purposes, when a patient's blood has been shown to be abnormal by the above simple screening tests, all that is required is an accurate haematological diagnosis. Usually this can be made without elaborate tests, often, in fact, by simple inspection of a blood

film. Without attempting to elaborate the point too far, the diagnostic signs to look for include the following: the intensity and uniformity of the staining of the red cells; their size and variation in size (anisocytosis); the degree to which they vary in shape (poikilocytosis); the presence of fragmenting cells (schistocytes), spherocytes or target cells, or immature red cells (giving rise to polychromasia); Howell-Jolly bodies or nucleated red cells; the various forms of punctate basophilia; the presence of autoagglutination or excessive rouleaux formation; excess or deficiency of the leucocytes—and the presence of abnormal or immature forms; and deficiency, excess or morphological abnormalities in the platelets. Quantitative counts and "absolute values" rarely give diagnostic information that cannot be appreciated from the inspection, by a trained observer, of a well spread and well stained blood film.

Nevertheless, it is desirable to obtain some quantitative data on venous blood: an estimation of the haemoglobin content or packed cell volume —or both to confirm perhaps a suspicion of iron deficiency—and a reticulocyte count are minimum requirements and should always be carried out; and in most instances, too, the totals of leucocytes and platelets should be counted, if there is any suspicion from the stained film that they may be abnormal.

Occasionally, no certain diagnosis is possible without bone-marrow biopsy. This aspect of diagnosis is considered separately in Chapter 7 It is, of course, also true in haemolytic and haemorrhagic disorders and in the megaloblastic anaemias, that the exact diagnosis can often be made only after detailed laboratory studies.

A total red-cell count rarely helps in diagnosis (for exceptions to this statement, see below); the error of the estimation (at least by visual counting methods) is unavoidably large (see p. 17), and calculation of the absolute values, even if the data can be relied upon (see p. 56), rarely gives information of decisive importance. Knowledge that the M.C.V is raised, for instance, does not help in differentiating a true megaloblastic anaemia from a haemolytic anaemia or from a macrocytic anaemia developing after haemorrhage Certainly a red-cell count usually gives less information than does a reticulocyte count

There are, however, two types of case in which an accurate red-cell count may be important in diagnosis:

(a) *In Polycythaemia.* In some patients, as a result of iron deficiency or haemorrhage, the haemoglobin and packed cell volume may be normal ... the red cell count may be grossly raised, however, the diagnosis is

and typically (in poly-

ts

... and packed

tation of bone-marrow films and sometimes even on more elaborate procedures such as vitamin B_{12} assay and studies with radioactive vitamin B_{12}

"COMPLETE" HAEMATOLOGICAL INVESTIGATION OF A PATIENT

Here it is assumed that the diagnosis has been made with certainty ›r with a high degree of probability and that the observer has sufficient leisure to investigate his patient as far as his resources allow, in order to confirm the diagnosis, or in an attempt to add to existing knowledge. Many measurements and tests can be carried out, e g , the accurate neasurement of absolute values, staining for siderocytes, or Heinz odies, the peroxidase reaction of leucocytes, examination of the bone-narrow by aspiration or biopsy, staining for marrow iron, estimation of serum iron or copper and of erythrocyte protoporphyrin or copro-porphyrin, spectroscopic study of the blood, study of the life-span of the patient's red cells or of donated red cells, serological studies, osmotic and mechanical fragilities, osmotic fragility after incubation, auto-haemolysis, electrophoresis of haemoglobin, solubility studies of haemo-lobin and resistance to alkali, serum vitamin B_{12} assay, studies with idioactive B_{12}, and electrophoresis of serum proteins While the results of many of these tests may help to clinch the diagnosis, most of them cannot be considered as routinely practicable Some should perhaps become so, most are best reserved for patients presenting some special problem when preliminary tests or the history suggest the pro-bable nature of the complaint

Examination of Blood Films

Earlier in this chapter the point was made that many blood disorders can be diagnosed by examination of stained films alone However, his can only be done if the films have been well spread and stained id if they are examined in a methodical way It is quite useless to place a drop of oil anywhere on the film and examine it directly with the high-power 2-mm objective

First, the film should be covered with a cover-glass using a neutral medium as mountant Next, it should be inspected under a low magni-fication (with the 16-mm objective) to get an idea of the quality of the preparation, and of the number, distribution and staining of the leucocytes, in this way also an area of even distribution where the red cells are not distorted can usually be found even in the worst made films.

Having selected a suitable field, the 4-mm objective should next be used A much better appreciation of variation in red-cell size, shape and staining can be obtained with this objective than with the 2-mm. oil-immersion lens The latter in combination with $\times$ 6 eyepieces should e reserved for the final examination of unusual cells and for looking fine details such as punctate basophilia, etc

As the diagnosis of the type of anaemia or abnormality present usually depends upon a comprehension of the whole picture which the film presents, the red cells, leucocytes and platelets should all be systematically examined, looking particularly for the abnormalities listed on p. 66. An eyepiece fitted with a micrometer scale and calibrated for use with the oil-immersion lens should be kept on the observer's bench. It is a useful accessory, as an impression of microcytosis or macrocytosis can be quickly confirmed by measuring the diameters of selected cells.

CHAPTER 6

SUPPLEMENTARY OPTICAL AND STAINING TECHNIQUES

EXAMINATION OF BLOOD CELLS IN PLASMA

The examination of a drop of blood sealed between a slide and cover-glass is sometimes of considerable value.

The preparation may be examined in several ways: by ordinary illumination, with or without the addition of dyes such as Janus green and neutral red (supravital staining); by dark-ground illumination, or by the phase-contrast technique. Chemically clean slides and cover-glasses should be used and the blood allowed to spread out thinly between them. If the glass surfaces are free from dust, the blood should spread out spontaneously and pressure, which is undesirable, should not be necessary. The edges of the preparation should be sealed with a melted mixture of equal parts of petroleum jelly and paraffin wax.

Rouleaux formation is usually seen in varying degrees in "wet" preparations of whole blood. The abnormal irregular rouleaux seen in haemolytic anaemias associated with marked spherocytosis are characteristic. *Autohaemagglutination* must be distinguished from rouleaux formation (see p. 202). This is important as the presence of true agglutination suggests the possibility of acquired haemolytic anaemia.

Blood parasites such as microfilariae and the sphirochaetes of relapsing fever may be seen in wet preparations of blood, the presence of small numbers of the latter being revealed by occasional slight agitation of groups of red cells. Sickling of the red cells may also be seen (see p. 132).

If a warm stage is available, the motility of the leucocytes can be studied, usually only the granulocytes show significant progressive movements. Whitby and Hynes (28) described the appearance of leucocytes under dark-ground illumination, cytoplasmic particles such as granules and mitochondria can be readily seen. Most workers, however, have studied preparations stained supravitally or have used the phase-contrast microscope. A description of the supravital technique is given below. Good illustrations of supravitally-stained cells are given in Whitby and Britton's (27) text-book.

Supravital Technique

Chemically clean glassware is essential. The following stains are required —

(*I*) A saturated solution of neutral red in absolute ethanol.

(*II*) A saturated solution of Janus green in absolute ethanol

Before use 0·2 ml. of (*I*) is added to 5 ml. of absolute ethanol and two 1·5-ml. volumes of this solution are delivered into separate test tubes As it is convenient to work with at least two concentrations of Janus green, 0·02 ml. and 0·05 ml. of solution (*II*) are added, respectively, to each of the two 1·5-ml. volumes of neutral-red solution.

Preparation of Slides. Several drops of the staining mixture are delivered by Pasteur pipette on to a clean slide so as to flood its surface; the excess is allowed to drain back into the tube and the slide is dried rapidly by waving it over a heated wire gauze. In this way an even deposition of the dye is obtained. When dry, slides may be stored until required, but they must be protected from dust.

"*Setting up*" *the Specimen.* A small drop of freshly drawn blood, taken directly from a skin puncture or from a vein, or a drop of bone-marrow aspirate, is placed on one or more prepared slides of each dye concentration and covered by a cover-glass so as to obtain a moderately thin film. The edges of the preparation are sealed with a mixture of equal parts of petroleum jelly and paraffin wax. Staining is slow and may need 20 to 30 minutes, it is hastened by keeping the slides at 37°C.

Janus green which stains the mitochondria blue is the more toxic of the [illegible]

bone-marrow preparations, stronger concentrations may be necessary to obtain good staining. The thickness of the film of blood also affects the staining power of the dyes

Neutral red is much less toxic, neutrophil granules are stained yellowish, those of eosinophils orange-red and those of basophils maroon Vacuoles may take up the stain, they appear as bright red spheres of varying size They tend to increase in size and number as the preparation is kept.

[illegible]

motility of lymphocytes, etc., can be observed in hanging-drop prepara- [illegible]

promonocyte and adult monocyte they are fairly numerous, but variable in size.

Specific Granules The staining reactions of the granules of the neutrophils (yellow), eosinophils (orange red) and basophils (maroon) have already been mentioned. Fine rose-coloured granules appear in the early types of promyelocytes and increase in number as the cells mature. They are most numerous in promyelocytes of medium maturity and may be closely packed in a large sector, in the more adult cells they are gradually replaced by the yellowish granules of the adult neutrophils.

Vacuoles staining bright red may be seen as small spheres in most adult lymphocytes, in monocytes they are characteristically present in rather large numbers. In both types of cell they vary in size and are present throughout the cytoplasm; a definite group (rosette) may be seen in the concavity of the nucleus of monocytes. Similar vacuoles are seen in promonocytes and monoblasts.

Erythroblasts Both fine mitochondria and vacuoles may be seen in developing red cells, but they diminish in number as the cells mature and the nuclei undergo pyknosis. Mitochondria are not seen in the adult red cell, an occasional red vacuole, however, may be encountered.

Phase-Contrast Microscopy

The introduction of the phase-contrast apparatus has provided new possibilities for the examination of biological material (4). In haematology it permits the observation of living cells and reveals their cytoplasmic structures without the use of potentially toxic dyes. In addition the nuclear chromatin and nucleoli of unstained cells can be visualized. Use of the phase-contrast technique has clearly demonstrated that mitochondria are responsible for the pale unstained areas so commonly seen in the cytoplasm of primitive cells, particularly close to the nuclear membrane (15) and that the nuclear pattern as seen in stained films is to some extent modified by the cytoplasmic structures that overlie it (16).

Phase-contrast microscopy combined with microcinematography gives a remarkable picture of the movements of living cells and of the intracellular constituents (see the recent review of Bessis (4) on phase-contrast microscopy (and electron microscopy) applied to blood cells). Good illustrations of the appearance of blood cells when viewed under phase-contrast are given by Bessis (3) and Ludin (18).

Value of Supravital Staining and the Phase-Contrast Microscopy in Clinical Haematology

These are primarily research techniques for they seldom reveal information of immediate practical importance. With supravital staining considerable care is needed to obtain good results, and both techniques require experience in interpretation. However, in addition to enabling observations of scientific rather than practical importance to be made, use of these techniques may help to differentiate the various types of acute leukaemia by providing more information than can be obtained

by Romanowsky or peroxidase staining. Although in the past this has always been considered a purely academic problem, the introduction of anti-metabolites and A.C.T.H. and cortisone in the treatment of leukaemia has increased the importance of accurate cytological diagnosis. The use of phase-contrast microscopy in counting platelets has already been referred to.

The Peroxidase Reaction Applied to Blood Films

This reaction depends upon the presence of enzymes (peroxidases) within the cytoplasm of leucocytes of the granulocyte series and to a lesser extent of monocytes. Blood or bone-marrow films, after fixation, are treated with benzidine and hydrogen peroxide and the benzidine is oxidized by the peroxide in the presence of peroxidase. Sodium nitroprusside which forms a blue compound with oxidized benzidine may be added to the benzidine, but it is not essential.

Solutions required—

I. Fixative· absolute ethanol, nine parts; neutral formalin (40% formaldehyde), one part.

II. A saturated aqueous solution of benzidine.

III. A saturated aqueous solution of sodium nitroprusside Before use one part of (*III*) is added to 99 parts of (*II*).

IV A freshly made solution of hydrogen peroxide " 10 vols " Two drops (0·04 ml.) are added to 10 ml. of distilled water.

Method. Fresh air-dried films are required. These are fixed for 1 minute in the formol-ethanol fixative solution, washed in distilled water and set upright to dry. One part of the peroxide solution is added to two parts of the nitroprusside-benzidine solution, and the slide is flooded with the mixture for 1 minute The slide is then washed in distilled water and examined under the microscope. If the staining is satisfactory, the granules in the neutrophils should be stained a deep blue-black. The preparation may be counterstained with 0·1% aqueous safranin for 1 minute or in dilute (1 in 20) Giemsa stain for 7 to 10 minutes

VALUE OF THE PEROXIDASE REACTION

Developing and adult granulocytes give a positive staining reaction, [illegible] Positively- [illegible] in promono- [illegible] re uniformly negative in both adult and immature cells

The peroxidase reaction is an interesting phenomenon, but is not often of much practical value. Occasionally, a positive reaction helps to distinguish between myeloblasts and lymphoblasts, but as the most immature myeloblasts give a negative reaction, the test usually breaks down just when it is most needed

ADDITIONAL STAINING METHODS

Unna-Pappenheim Stain

The Unna-Pappenheim combination of pyronin with methyl green can be used to stain both dry- or wet-fixed films of peripheral blood or aspirated bone-marrow Wet-fixed films give the more satisfactory results. The pyronin stains bright red the cell components containing ribonucleic acid, e g , the nucleoli and the cytoplasm of all types of primitive cells and of mature cells, such as plasma cells, in which the ribonucleic acid persists. The methyl green stains the chromatin greenish black.

[illegible]

tion —

Pyronin (B D H) .	0 3 g.
Methyl green	0 7 g
Glycerin	20 ml.
Absolute ethanol	2·5 ml.
0 5% phenol in distilled water to 100 ml.	

The stains are ground with the glycerin and ethanol in a mortar and the phenol-water added later The solution is boiled for 2 minutes then filtered

After staining, the slides are rinsed in distilled water and dehydrated in tertiary-butanol, cleared in xylol and mounted in neutral balsam or Gurr's medium If differentiation is required, a rapid dip into absolute ethanol, in which pyronin is very soluble, suffices Dehydration in the butanol is [illegible]

which in wet- elongated red n dry films the , ie In certain respects wet-fixation has definite advantages over dry-fixation Delicate cells and structures are often better preserved, and in conjunction with ribonuclease the stain can be used as a histochemical test In wet-fixed films leucocytes are more globular, i e , more nearly the shape in life The red cells are badly distorted as a rule

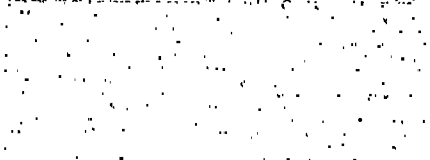
Feulgen's Stain

The Feulgen reaction is generally held to be a specific cytochemical test for desoxyribonucleic acid, an important component of nuclear chromatin (22) When applied to films of bone-marrow or peripheral blood the nuclei of the adult cell types stain most intensely, and the nuclei of the primitive cells least intensely. The nucleoli do not stain. Howell-Jolly bodies stain by this method, i e , they are Feulgen-positive,

[illegible] 4 ml

and chromosomes stain particularly well. The stain is thus well suited to the study of mitoses.

The best staining results are obtained after wet-fixation with Susa's fluid, but satisfactory staining of dry-fixed films can be achieved. In a well stained preparation the nuclear chromatin is sharply stained shades of purple.



water for 10 minutes, rinsed in distilled water and counterstained by immersion for a few seconds in a 1% aqueous solution of light green.

The chemistry of the Feulgen reaction is complex and has been the subject of some controversy (22).

Staining with Sudan Black

Sudan black B. has been used by Sheehan (25) and later by Baillif and Kimbrough (1), and others, to stain the granules of leucocytes, many of which appear to contain lipoid. The results indicate a close parallelism between sudanophilia and a positive peroxidase reaction, and Sudan-black staining may perhaps be used as a substitute for the enzyme reaction. Further details and references to the literature are given in the above-mentioned papers, and by Rheingold and Wislocki (23)

THE STAINING OF SIDEROCYTES

Siderocytes, or red cells containing granules of iron which give a positive Prussian-blue reaction, were described by Gruneberg (13) in small numbers in the blood of normal rat, mouse and human embryos, and in large numbers in mice with a congenital anaemia. They have since been detected in adult human blood, chiefly after splenectomy. They are found in nucleated as well as in non-nucleated red cells, but only in those in which haemoglobin is being formed. The granules also stain by Romanowsky dyes (7, 19, 21) (Fig. 10).

It now seems that siderotic granules are to be found normally in many of the normoblasts of human bone-marrow and in marrow reticulocytes (8, 17). The exact reason for this is not yet clear; possibly the

... of basic fuchsin in 200 ml of ... 50 C, ... ydrous ... low; it ... 4 hours after preparation

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siderotic granules of a normoblast represent iron taken into the cell in excess of that required for immediate incorporation into haem. Certainly, siderotic granules far larger than normal are found in erythroblasts when there is impaired synthesis of haemoglobin—as in Mediterranean anaemia and similar syndromes (8), and in lead poisoning (20). After splenectomy, siderocytes are nearly always found in the peripheral blood, often in large numbers, but the exact role of the spleen in relation to the numbers of circulating siderocytes is uncertain (6).

Method of Staining

Air-dried films are fixed with methanol for 10 to 20 minutes. When dry, the slides are placed in a solution of 1% potassium ferrocyanide in 0·1N-HCl, made by mixing equal volumes of 2% potassium ferrocyanide and 0·2N-HCl immediately before use. The most consistent results are obtained by carrying out the staining in a water-bath at 50° to 56°C.

After 10 minutes the slides are well washed in running tap water for 20 minutes, rinsed in distilled water and then weakly counterstained with 0 1% aqueous safranin or 0·1% aqueous eosin for a few seconds.

Siderocytes contain one or more (rarely many) iron-containing unevenly distributed granules, varying in size from about 1·5 μ. down to the limits of visibility (Fig 10)

HEINZ BODIES IN RED CELLS

Heinz (14), in 1890, was the first to describe in detail inclusions in red cells developing as the result of the action of acetylphenylhydrazine on the blood. Now it is known that " Heinz " bodies may be produced by the action on the blood of a wide range of aromatic nitro- and amino- [illegible]

pheral blood if the patient has undergone splenectomy.

Heinz bodies probably consist of denatured globin derived from haemoglobin. Methaemoglobin is not usually formed under conditions which favour Heinz-body formation (2) Recent reviews dealing with Heinz bodies include those of Webster (26), Buckell and Richardson (5), and Fertman and Fertman (10)

Demonstration of Heinz Bodies

Unstained Preparations. Heinz bodies may be seen as refractile objects in dry unstained films, if the illumination is cut down by lowering the microscope condenser. They are also easily seen by dark-ground illumination or phase-contrast microscopy The size of the particles

varies from 1 to 2 μ. to half the size of the corpuscles One or more may be present in a single cell. They are usually close to the cell membrane, and in wet preparations may move around within the cells in a slow Brownian movement.

Stained preparations. Methyl violet stains the bodies excellently. Equal volumes of blood and 0·5% methyl violet in normal saline are mixed together and the suspension allowed to stand for about 10 minutes at room temperature. Films may then be made or the suspension of corpuscles viewed between slide and cover-glass. The Heinz bodies stain an intense purple (Fig. 11). They also stain with other basic dyes. With brilliant cresyl blue they stain less intensely than with methyl violet. However, they may be readily seen as pale blue bodies in a well stained reticulocyte preparation, if the preparation is not counterstained.

If permanent preparations are required, the vitally stained films should be fixed by exposure to formalin vapour for 5 to 10 minutes. If films are fixed in methanol, the bodies are decolorized. Formalin-fixed films may be counterstained with 0 1% eosin or 0 1% safranin after thoroughly washing in distilled water.

STAINING THICK BLOOD FILMS

The thick blood film is widely used in the diagnosis of malaria. By this technique a relatively large volume of blood may be scrutinized in a short time and parasites seen even if present in very small numbers. Field's method of staining (11) is quick and usually satisfactory. This method and the modification proposed by Fenton and Innes (9) will be described.

Making Thick Films

Thick films are made by placing a small drop of blood in the centre of a slide and spreading it out with a corner of a slide to cover an area about 15 mm. in diameter. The film is then allowed to dry thoroughly for 30 minutes at 37 C before staining is attempted. Absolutely fresh films, although apparently dry, may wash off in the stain

Field's Method (11)

Stain A (polychromed methylene blue)

Methylene blue	1 3 g
Disodium hydrogen phosphate (Na_2HPO_4 $12H_2O$)	12 6 g.
Potassium dihydrogen phosphate (KH_2PO_4)	6 25 g.
Distilled water	500 ml.

The methylene blue and the disodium hydrogen phosphate are dissolved in 50 ml of distilled water The solution is then boiled and evaporated in a water-bath almost to dryness in order to "polychrome" the dye The potassium dihydrogen phosphate and 500 ml. of freshly boiled distilled water

[illegible]

then be dissolved directly in the phosphate buffer solution. No evaporation is necessary.

Stain B (eosin)

Eosin	1 3 g
Disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$)	12 6 g
Potassium dihydrogen phosphate (KH_2PO_4)	6·25 g.
Distilled water	500 ml.

The phosphates are dissolved in warm freshly boiled distilled water, and the dye then added The solution should be filtered after standing for 24 hours.

Method of Staining

The slide carrying the dried but otherwise unfixed film is dipped into Stain *A* for 1 to 2 seconds. It is then rinsed in buffered distilled water (pH 6 8 to 7 0) until the stain ceases to flow from the film (5 to 10 seconds) Next it is dipped into Stain *B* for 1 to 2 seconds and *then rinsed rapidly (1 to 2 seconds) in buffered distilled water* The slide is finally set upright to dry after the excess water has been shaken off, it must not be blotted

Fenton and Innes's Modification (9) of Field's stain

Three staining solutions are required.

Stain A (polychromed methylene blue)

Solution I.	Methylene blue	1 g
	Disodium hydrogen phosphate (Na_2HPO_4 $12H_2O$)	5 g
	Distilled water	250 ml

[illegible] laced in [illegible] hours to [illegible] When [illegible]

Solution II	Methylene blue	1 g
	Potassium dihydrogen phosphate (KH_2PO_4)	2 g
	Distilled water	500 ml.

Two parts of Solution II are added to one part of Solution I to give Stain *A* The addition of Solution II assures that some unchanged methylene blue is present in the mixture

Stain B (eosin)

[illegible]	1 g
[illegible]	3 3 g
[illegible]	1 3 g.
[illegible]	500 ml

The pH of both stains should be 6 8.

varies from 1 to 2 μ. to half the size of the corpuscles. One or more may be present in a single cell. They are usually close to the cell membrane, and in wet preparations may move around within the cells in a slow Brownian movement.

Stained preparations. Methyl violet stains the bodies excellently. Equal volumes of blood and 0 5% methyl violet in normal saline are mixed together and the suspension allowed to stand for about 10 minutes at room temperature. Films may then be made or the suspension of corpuscles viewed between slide and cover-glass. The Heinz bodies stain an intense purple (Fig. 11). They also stain with other basic dyes. With brilliant cresyl blue they stain less intensely than with methyl violet. However, they may be readily seen as pale blue bodies in a well stained reticulocyte preparation, if the preparation is not counterstained.

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Disodium hydrogen phosphate (Na_2HPO_4 $12H_2O$) ..	12 6 g.
Potassium dihydrogen phosphate (KH_2PO_4) .. .	6 25 g.
Distilled water	500 ml.

The phosphates are dissolved in warm freshly boiled distilled water, and the dye then added The solution should be filtered after standing for 24 hours

Method of Staining

The slide carrying the dried but otherwise unfixed film is dipped into Stain *A* for 1 to 2 seconds It is then rinsed in buffered distilled water (pH 6·8 to 7 0) until the stain ceases to flow from the film (5 to 10 seconds) Next it is dipped into Stain *B* for 1 to 2 seconds and then rinsed rapidly (1 to 2 seconds) in buffered distilled water. The slide is finally set upright to dry after the excess water has been shaken off; it must not be blotted

Fenton and Innes's Modification (9) of Field's stain

Three staining solutions are required

Stain A (polychromed methylene blue)

Solution I	Methylene blue	1 g
	Disodium hydrogen phosphate (Na_2HPO_4 $12H_2O$)	5 g.
	Distilled water	250 ml.

[illegible]

Solution II.	Methylene blue	1 g.
	Potassium dihydrogen phosphate (KH_2PO_4)	2 g
	Distilled water .	500 ml.

Two parts of Solution II are added to one part of Solution I to give Stain *A* The addition of Solution II assures that some unchanged methylene blue is present in the mixture

Stain B (eosin)

[illegible]	1 g.
	3 3 g
	1 3 g
	500 ml.

The pH of both stains should be 6 8

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Potassium dihydrogen phosphate (KH_2PO_4)	6 25 g.
Distilled water	500 ml

... hydrogen phosphate are dissolved
...vaporated in a
... the dye The
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is necessary

Stain B (eosin)

Eosin	1·3 g.
Disodium hydrogen phosphate (Na_2HPO_4 $12H_2O$)	12 6 g.
Potassium dihydrogen phosphate (KH_2PO_4)	6·25 g.
Distilled water	500 ml.

The phosphates are dissolved in warm freshly boiled distilled water, and the dye then added The solution should be filtered after standing for 24 hours.

Method of Staining

The slide carrying the dried but otherwise unfixed film is dipped into Stain *A* for 1 to 2 seconds It is then rinsed in buffered distilled water (pH 6 8 to 7 0) until the stain ceases to flow from the film (5 to 10 seconds). Next it is dipped into Stain *B* for 1 to 2 seconds and then rinsed rapidly (1 to 2 seconds) in buffered distilled water. The slide is finally set upright to dry after the excess water has been shaken off; it must not be blotted

Fenton and Innes's Modification (9) of Field's stain

Three staining solutions are required

Stain A (polychromed methylene blue)

Solution I	Methylene blue	1 g.
	Disodium hydrogen phosphate (Na_2HPO_4 $12H_2O$)	5 g.
	Distilled water	250 ml

The methylene blue, sodium phosphate and distilled water are placed in

Solution II	Methylene blue	1 g.
	Potassium dihydrogen phosphate (KH_2PO_4)	2 g
	Distilled water	500 ml.

Two parts of Solution II are added to *one* part of Solution I to give Stain *A* The addition of Solution II assures that some unchanged methylene blue is present in the mixture

Stain B (eosin)

Stain C (dilute Leishman's stain)

Buffer solution

Disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$) ..	12·5 g.
Potassium dihydrogen phosphate (KH_2PO_4)	5·0 g.
Distilled water	250 ml.

The pH of this solution should also be 6·8.

Leishman's stain (p. 38) is diluted 1 in 50 in distilled water and 3 parts per 100 of the above buffer added.

Method of Staining

[illegible]
for 10 to 15 minutes. They are then stood upright to dry without further washing.

Successfully stained films should be faint purple-blue in colour, transparent and contain no residual haemoglobin. The leucocytes, platelets and parasites should stand out against a colourless background. The nuclei of the leucocytes should be well preserved and stained. The cytoplasm of the parasites should be stained blue or grey-blue and *the chromatin dot a bright red. Schuffner's dots, if present, should* stain intensely.

Fenton and Innes's method, although a little more elaborate, seems to give more consistently good results than does Field's method.

Relative Value of Thick and Thin Films in the Diagnosis of Malaria

Thick films are extremely useful when parasites are scanty, but the identification of the parasites is less easy. Mixed infections may be missed and there may be doubt as to the identification of any particular object. However, an experienced observer may be able to find and recognize with certainty parasites even in badly stained thick films, whilst in a well stained film parasites should be easily recognized, even by beginners. Five minutes spent in examining a thick film is equi- [illegible] doubt as to whether or not an object is a [illegible] is severe leucopenia. [illegible] least to estimate the proportion of polymorphonuclear to mononuclear cells) much more rapidly and more accurately than in thin films made from the same blood (see also p. 46).

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CHAPTER 7

BONE-MARROW BIOPSY

BIOPSY of bone-marrow is an indispensable adjunct to the study of diseases of the blood and may be the only way in which a correct diagnosis can be made. The method of surgical biopsy of the sternum, ilium, tibia or rib has been largely superseded, but not quite replaced, by needle (aspiration) biopsy of the sternum or other bones. Needle biopsy has many advantages over the surgical method; it is simple, safe, and relatively painless, and it can be repeated many times and even performed on out-patients Not only does it provide material for making films but quite good sections of aspirated marrow fragments can be obtained. Surgical biopsy has several disadvantages and can seldom be repeated (see p 84) However, it is capable of giving decisive information when methods of aspiration fail and it has thus a definite place in diagnosis Bone-marrow puncture seems to be safe in almost all circumstances, even in thrombocytopenic purpura. However, it should never be attempted when there is a major disorder of coagulation as in haemophilia.

Percutaneous trephine biopsy is a compromise between the aspiration and surgical-biopsy techniques, and combines some of the advantages of both. Either " microtrephines " of 2-mm. bore or less (4, 26) or larger trephines, e g, that of Sacker and Nordin (25) which has an 8-mm. bore, can be used The small microtrephines can be inserted into the sternum, vertebral spines or iliac crest, but the larger trephines should be used only on the iliac crest (see p 85) The various methods that can be used for aspiration and biopsy of bone-marrow have been reviewed recently by Berman (2)

TECHNIQUE OF NEEDLE (ASPIRATION) BIOPSY OF THE BONE-MARROW

Sternal Puncture

The usual site for puncture is the manubrium or the first or second pieces of the body of the sternum The manubrium contains rather denser trabecular bone than does the body of the bone and, in elderly subjects at least, tends to contain more fatty marrow than is found elsewhere in the sternum (7) It is also sometimes less easy to be certain that the needle point has reached the cavity of the bone However, completely satisfactory samples are obtained more often than not from the manubrium and there is the advantage that as the bone

is punctured well above the level of the heart the consequences of accidental transfixion of the [illegible]

[illegible] mage resulting from previous punctures.

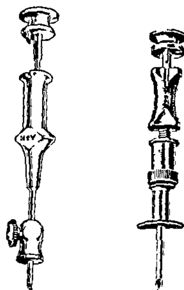


FIG 12 SALAH (LEFT) AND KLIMA (RIGHT) MARROW-PUNCTURE NEEDLES (REDUCED × ⅔)

(Reproduced from *Disorders of the Blood* by Sir Lionel Whitby and Dr C J C Britton (1950), 6th Ed, p 704 Churchill, London)

A special needle is used for sternal puncture (Fig. 12). This should be short and stout with a well fitting stilette, and must be provided with an adjustable guard The patient lies on his back in a semi-recumbent position and the skin covering the upper part of the sternum is cleansed with spirit or iodine If hairy, the site must be shaved. The skin, subcutaneous tissue and periosteum overlying the site selected for the puncture are carefully infiltrated with a local anaesthetic such as Xylocaine.

If the manubrium is selected, the site of the puncture should be about 1 cm. above the sterno-manubrial angle and slightly to one side of the mid-line: if the body of the bone is to be punctured, this should be done opposite the second or third intercostal spaces slightly to one side of the mid-line.

The guard on the needle is adjusted to about 12 to 15 mm. from the needle point, depending on the thickness of the subcutaneous tissue. The needle is then pushed with a boring motion into the cavity of the

bone. The amount of force required varies, but may need to be considerable. It is usually easy to appreciate when the cavity of the bone has been entered. The stilette is then removed and a well fitting 1- or 2-ml. syringe used to suck up not more than 0·3 ml. of marrow contents—bone-marrow diluted with a variable amount of blood. As a rule material can be sucked into the syringe without difficulty; occasionally it may be necessary to re-insert the stilette and to push the needle in a little farther and to suck again. If further attempts at aspiration prove unavailing, it is worthwhile trying the effect of greater suction; a 5- or 10-ml. syringe may be used for this purpose.

Films should be made from the aspirated material without delay (see p. 86). The remainder of the material may then be delivered into a suitable fixative for histological sections (see p. 88). The films should be stained as soon as dry by the May-Grünwald-Giemsa technique, or fixed for 10 to 20 minutes in absolute methanol if it is necessary to postpone staining for longer than a few hours.

Spinous-Process Puncture

The spinous processes of the vertebrae can be punctured and good samples of marrow may be obtained from adults (15). Puncture is not difficult, since the bones lie superficially, but rather more pressure is required than for sternal puncture. The needle should be passed into the spine of a lumbar vertebra slightly lateral to the mid-line in a direction at right angles to the skin surface, with the patient either sitting up or lying on his side as for a lumbar puncture. Advantages of the method are that the patient cannot see what is happening and that several attempts at puncture and aspiration can be made, if necessary, in the same anaesthetized area.

Iliac-Crest Puncture

The iliac crest is another site from which active marrow may be withdrawn (24). The needle should be passed into the cavity of the ilium perpendicular to the flat surface, 2 cm posterior and 2 cm. inferior to the anterior superior iliac spine. As with spinous-process puncture the bone is often appreciably harder to pierce than is the sternum, [illegible]

Comparison of the Different Sites for Needle Puncture

Although there is normally considerable variation in the composition of cellular marrow withdrawn from adjacent or different sites, the general trend and type and maturity of haemopoiesis and the balance between erythropoiesis and leucopoiesis are similar (9, 17). In practice it is a distinct advantage to have a choice of several sites for puncture,

particularly when puncture at the usual site results in a "dry tap" or when blood alone is withdrawn. Aspiration at a different site may yield cellular marrow or strengthen suspicion of a widespread change affecting the bone-marrow, such as fibrosis or aplasia.

The actual type of aspirating needle used is a matter of personal preference. However, only needles designed for the purpose should be used. They should be of hard stainless steel, about 7 to 8 cm. in length with an outside bore of about 1·5 mm. They must be provided with a well fitting stilette and have a guard the position of which can be adjusted on a spiral thread or kept in position by a lateral screw (Fig 12). The point of the needle and the edge of the bevel must be kept well sharpened.

A novel type of needle has been described recently by Reddy (23) It has a lateral cutting edge designed to cut out a solid piece of marrow when the needle is rotated Reddy found the needle to be particularly useful in providing material for the demonstration of Leishman-Donovan bodies.

Needle Biopsy of the Bone-Marrow in Children

In the youngest children, from birth to 2 years, the medial aspect of the upper end of the tibia just below the level of the tibial tubercle may be punctured and active marrow withdrawn. In older children the tibial cortical bone is usually too dense and the marrow within normally less active. Iliac puncture is then the method of choice. Sternal puncture, although possible, is not free from danger for the bone is thin and the marrow cavities small. The dimensions of the marrow cavities in the sternum of children are given by Diwany (8)

Marrow Puncture in Laboratory Animals

Details of techniques are described by McFadzean (16) It is suggested that the bone (posterior iliac spine) be drilled with a dental drill and bone-marrow aspirated through the hole thus made, using a fine needle attached to a small syringe

SURGICAL BIOPSY OF THE BONE-MARROW

As already mentioned, surgical biopsy of the bone-marrow has several disadvantages. the biopsy has to be carried out in an operating theatre using a full aseptic technique and it can seldom be repeated; it may be dangerous if the patient has a bleeding diathesis or granulocytopenia, and in leukaemia, particularly, the incision may fail to heal. On the other hand, the method has the advantage of providing a relatively large amount of bone-marrow tissue from which, after decalcification, sections may be cut The method is thus particularly suitable for use in the demonstration of myelofibrosis, osteosclerosis or metastatic tumour deposits, conditions in which it may prove impossible to

obtain satisfactory specimens by aspiration. For the study of cytology it is, of course, possible to prepare films or imprint preparations from the material obtained by biopsy.

Biopsy may be carried out on the sternum, iliac crest or rib. However, as biopsy of the sternum often leaves an ugly scar which the patient can see, the latter two sites seem preferable Particularly good sections can be obtained if a short segment of a rib is removed (10).

PERCUTANEOUS TREPHINE BIOPSY OF THE BONE-MARROW

Türkel and Bethell (26) described a microtrephine of about 2-mm bore which could be passed through a hollow introducing needle only slightly larger than a marrow aspirating needle No skin incision is necessary and the instrument can be safely used on the sternum. A rather similar instrument for combined biopsy and aspiration was described later by Bernstock and Sterndale (4) However, the cylinders of bone and underlying marrow obtained by these trephines are small, and they are apt to break up while being prepared for sectioning. Larger trephines seem to be more valuable in practice, and their use is hardly more disagreeable to the patient The Sacker-Nordin trephine (25) (Fig. 13), which can be safely used on the iliac crest, provides as a rule sufficient material for an accurate diagnosis (Figs 14 and 16) The instrument can be inserted under local anaesthesia, but as a small skin incision is necessary the biopsy is best performed in the operating theatre where full aseptic precautions can be taken

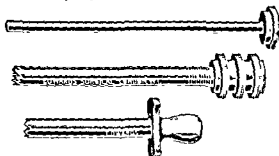


FIG 13 TREPHINE FOR BONE OR BONE-MARROW BIOPSY, DESIGNED BY DRS L S SACKER AND B E C NORDIN
(Reproduced from the *Lancet*, I, 347, 1954)

EXAMINATION OF ASPIRATED BONE-MARROW

The volume of marrow which may be aspirated by puncture is limited, and the more material aspirated the greater is the proportion of contaminating blood; hence there is for most purposes little if any advantage in aspirating more than 0 2 ml. of marrow fluid.

The material aspirated can be dealt with in at least four ways: (a) films can be made of the material as aspirated; (b) films can be made after concentration; (c) "particle smears" can be made, and (d) histological sections can be cut.

Bone-Marrow Films

Careful preparation is essential and it is desirable, if possible, to concentrate the marrow cells at the expense of the blood by which they are diluted.

The following simple device is generally satisfactory. Drops of the aspirate are delivered on to the slides about 1 cm. from one end and most of the blood is then sucked off with a fine Pasteur pipette applied to the edge of each drop. The irregularly shaped marrow fragments tend to adhere to the slide and most of them are left behind. A film 3 to 5 cm. in length is then made of the marrow fragments and the remaining blood by means of a smooth-edged glass spreader of not more than 2 cm. in width (Fig. 15). The marrow fragments are dragged behind the spreader and leave a trail of cells behind them. (It is in

become incorporated in a differential count.)

The preparation can be considered satisfactory only when marrow particles as well as free marrow cells can be seen in stained films, as is usual with the above technique. No attempt is made to squash the marrow particles. Their structure—whether hypocellular or hypercellular—can be readily appreciated without recourse to squashing, and their iron content revealed by staining with potassium ferrocyanide in acid solution (see p. 75).

Bone-marrow films can be fixed and stained with Romanowsky dyes by exactly the same techniques as for peripheral blood films (p. 39). Thorough fixation (at least 20 minutes in methanol) is essential if high-quality staining is required.

Concentration of Bone-Marrow by Centrifugation

Several workers have used centrifugation techniques in an attempt to concentrate the marrow cells and to assess the relative distribution of marrow cells, peripheral blood and fat in aspirated material. Limarzi (14), for instance, devised a method for centrifuging heparinized aspirated marrow in a Wintrobe haematocrit tube. "Yellow fat", "red fat", plasma, marrow cells and mature red cells separate from above downwards. The marrow-cell layer is used for preparing films and for differential counts.

Although concentration of poorly cellular samples is useful, it is unnecessary when the aspirated material is of average or increased

Fig. 14. Biopsy Specimen of Human Bone-Marrow from Iliac Crest (natural size), obtained using a Sacker-Nordin Trephine

Fig 15 Film of Aspirated Bone-Marrow

The marrow particles are easily visible, mostly at the tail of the film (natural size).



Fig 16 Biopsy Specimen of Bone Marrow ×8 5
Same specimen as shown in Fig. 14 On the left is periosteum and on the right bone and bone-marrow

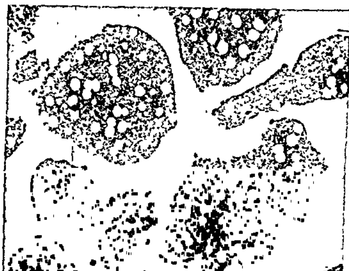


FIG 17 SECTION OF ASPIRATED BONE-MARROW PARTICLES PREPARED BY THE METHOD OF RAMAN (1955) × 60

cellularity. The presence of an anticoagulant may affect the quality of the subsequent staining and the delay before films are made results in some degeneration of the leucocytes. Moreover, while volumetric data are of value in group studies they are of less significance in individual patients because of the wide range of values encountered even in the normal.

"Particle Smears"

Some workers deliberately isolate aspirated marrow particles and make "smears" of them on slides or between two cover-slips using slight pressure (20). While this technique undoubtedly gives preparations of authentic marrow cells, the smearing out of the particle causes disruption and distortion of cells, and the resultant thick preparations are difficult to stain really well. The author feels that this technique has no advantages over the method described on p. 86.

The Preparation of Histological Sections of Aspirated Bone-Marrow

The advantage of sectioned material is that a slightly better picture of the marrow architecture is obtained than can be deduced from film of bone-marrow. The proportion between cellular marrow and fa spaces is preserved, hypoplasia or hyperplasia can be recognized, and invasion by tumour or the presence of granulomatous lesions can be seen. Nevertheless, for cytological detail, sectioned material is much less satisfactory. The subtle differences between cells such as normoblasts and megaloblasts, which are usually easy to appreciate in well stained films, are difficult to recognize in sections and it may sometimes be difficult even to differentiate erythroblasts from leucocytes with complete certainty—particularly is this true of trephine or surgical biopsy material which usually needs decalcification.

The fragments of bone-marrow aspirated by the puncture technique are small, rarely greater than 1 mm in size, and a careful technique in handling them is required. They are usually free from bone and the marrow architecture is well preserved, but their usefulness is limited because their small size makes it uncertain how representative of the bone-marrow they are. A more serious disadvantage of the technique is that fragments are often not obtained by suction in just those patients—with perhaps marrow aplasia, fibrosis or invasion by tumour—in whom histological evidence of any marrow abnormality is most urgently required. In these patients trephine biopsy may be necessary.

A number of methods of dealing with aspirated fragments have been published (2, 5, 22) which differ in the details of handling and concentrating the fragments, fixation and embedding. The following method gives an adequate concentration of the marrow particles and is simple to carry out (Fig. 17). Fixation is good and sections may be successfully stained by Romanowsky dyes as well as by standard methods.

A Method of Preparing Histological Sections of Aspirated Bone-Marrow Particles (Raman (22))

Fixative

Absolute ethanol is diluted with an equal volume of 15% (v/v) formalin. The mixture has a specific gravity of 0·93, almost exactly the same as that of human fat. When a marrow aspirate is added to this fixative, the blood remains in suspension while the marrow particles rapidly sediment. Even fatty marrow settles down in a few seconds.

Method

0·25 ml. of bone-marrow aspirate is added to 20 ml. of the fixative in a screw-capped bottle, and thoroughly mixed. It is then allowed to fix overnight.

The following morning, the sediment is resuspended by inversion The coarser marrow fragments are then picked out with a Pasteur pipette and teat, after they have re-settled to the bottom of the bottle, which usually takes only a few seconds They are then transferred to a centrifuge tube provided with a rubber bung, and containing 70% (v/v) ethanol. The material is subsequently dehydrated with two changes of absolute ethanol, being left for 1 hour in each. The ethanol is drained off and replaced with a 2·5% solution of collodion (Necol) in equal parts of absolute ethanol and ether, and left overnight. The embedding in Necol is necessary to hold the fragments together before they are finally embedded in paraffin wax.

On the third day, the excess Necol is drained off and replaced with 2 to 3 ml of chloroform to harden the collodion After 30 seconds the aggregated mass of fragments in the Necol is displaced by gently warming the tip of the tube over a very weak flame. The mass of fragments is then cleared with two changes of benzene and embedded in paraffin wax

A Method of Staining Sections of Bone-Marrow by May-Grunwald-Giemsa Stain

The many different techniques which are in use for staining by Romanowsky dyes are evidence of the real difficulty in obtaining satisfactory staining of sectioned material

The following method gives quite good results. It may be applied to aspirated marrow fragments, trephine or postmortem material. If decalcification is necessary, the results are less satisfactory. Thin pieces of the biopsy material are fixed as soon as possible in Helly's fluid* for 12 to 18 hours. They are then washed in running water overnight before being decalcified (should this be required), dehydrated and embedded in paraffin wax in the usual way. The sections when cut

* Helly's fluid consists of potassium dichromate, 2 5 g , mercuric chloride, 5 g., formalin (40% formaldehyde), 5 ml , distilled water, 100 ml

should be first placed in Lugol's iodine for 2 minutes and then in 5% sodium thiosulphate for 2 minutes They are washed in several changes of distilled water and then rinsed in distilled water buffered to pH 6·8

The sections are stained for 1 hour in May-Grunwald stain diluted with an equal volume of buffered distilled water, and then transferred without washing into Giemsa stain diluted with 19 volumes of buffered distilled water and left for 2 hours. The sections become grossly overstained and deep blue in colour, they are rinsed in buffered distilled water before differentiation.

The sections are differentiated by covering with a small volume of glycerin-ether (Gurr) freshly diluted with four parts of absolute ethanol. Differentiation takes place quickly and is usually adequate in a few seconds. The section is next dehydrated by a rapid dip in absolute ethanol, cleared in xylol and finally mounted in Gurr's neutral mounting medium The use of glycerin-ether helps to prevent " blueing " of the section during dehydration

In a successfully stained section the cytoplasm of primitive cells should be blue, that of myelocytes and segmented neutrophils pale pink, the eosinophil granules should be bright red and the cytoplasm of the red cells orange Neutrophil granules are usually not easily visible

Quantitative Cell Counts on Aspirated Bone-Marrow

A number of figures for the cell content of aspirated normal marrow have been given in the literature (21, 27) That the variation is extremely wide is hardly surprising, in view of the tendency of the marrow cells to adhere in clumps of varying size and the uncontrollable factor of dilution with peripheral blood, which according to Berlin, Hennessy and Gartland (1) may amount to 40 to 100% in 0 25- to 0 5-ml bone-marrow samples

For the above reasons quantitative cell counts seem hardly worth carrying out The degree of cellularity can be assessed within broad limits as *increased*, *normal* or *reduced* by inspection of a stained film containing marrow particles (see also p 86), and for practical purposes this seems all that is necessary

Differential Cell Counts on Aspirated Bone-Marrow; the " Myelogram "

Most workers perform differential counts on marrow films and by presenting the data in the form of a myelogram express the incidence of the various cell types as percentages Such figures are unfortunately not as accurate as they might appear It must not be forgotten that films made from aspirated material include cells from the peripheral blood as well as from the bone-marrow, and that the varying dilution with blood involves an error for which no compensation is possible. In addition, the more fixed and primitive cells, and large cells such as

megakaryocytes, tend to resist aspiration and remain in the marrow or embedded in marrow fragments Those which are aspirated tend to be most irregularly distributed; many are carried to the tail of the film.

Pontoni (19) introduced the term "haemomyelogram" for a differential count performed on aspirated material and restricted the use of the "myelogram" to differential counts made when fully ripened leucocytes are excluded, a more correct usage. His leuco-erythrogenetic ratio based on his "myelogram" is similarly a better expression of the relative proportions of leucopoiesis to erythropoiesis than is the more commonly used myeloid-erythroid ratio which is based on counts from which mature leucocytes are not excluded (5).

Ideally, differential counts should be performed on sectioned material, but the great difficulties in identification make this impractical Fadem and Yalow (9) recommended that differential counts be done on preparations made by the particle-smear technique. As mentioned on p. 86, a fairly reliable method is to count the cells in the trails of cells left behind the marrow particles as they are carried to the tail when a film is spread. Dameshek, Henstell and Valentine (6) gave interesting figures for parallel counts made on sections and on smears, and other observations are given in Osgood and Seaman's review (21). Osgood and Seaman also discussed the unavoidable statistical errors involved in performing differential counts, and the necessity for counting large numbers of cells, particularly if an attempt is being made to assess the frequency of cells present in only small numbers. A further difficulty is the impossibility of accurately dividing into arbitrary classes cells of which every gradation of development may be seen

For all the reasons referred to above, and because of the naturally variegated pattern of the bone-marrow and the irregular distribution of the marrow cells when spread in films, differential cell counts on marrow aspirated from normal subjects indicate a very wide range of normality, a range so wide that minor degrees of deviation from the normal occurring in disease are difficult to establish.

The normal values given in Table VII are based upon the data of several authors (9, 11, 12, 13, 18, 21) They can only be taken as an approximate guide. The proportion of fatty to cellular marrow in the sternum of healthy adults was given by Berman and Axelrod (3) as 21 to 52%.

Reporting on Bone-Marrow Films

The first thing to do is to look with the naked eye at a selection of slides and to choose from them several of the best spread films containing easily visible marrow particles The particles should then be examined with a low-power (16-mm.) objective with particular reference to their cellularity, an estimate of whether the marrow is hypoplastic, normoplastic or hyperplastic can usually be made without much difficulty, if sufficient particles are available for study The next

TABLE VII

NORMAL RANGES FOR DIFFERENTIAL COUNTS ON ASPIRATED BONE-MARROW (9, 11, 12, 13, 18, 21)

Reticulum cells	0 1–2%
Haemocytoblasts	0 1–1%
Myeloblasts	0 1–3·5%
Promyelocytes	0 5–5%
Myelocytes	
neutrophil	5–20%
eosinophil	0 1–3%
basophil	0–0 5%
Metamyelocytes	
young forms } stab forms }	10–30%
Polymorphonuclears	
neutrophil	7–25%
eosinophil	0 2–3%
basophil	0–0 5%
Lymphocytes	5–20%
Monocytes	0–0 2%
Megakaryocytes	0 1–0 5%
Plasma cells	0 1–3 5%
Pronormoblasts	0 5–5%
Normoblasts	
polychromatic	2–20%
pyknotic*	2–10%
Myeloid-erythroid ratio	2 5–15 1

* The term "pyknotic" is preferred to "orthochromatic" as a description of the most mature normoblasts Cells with fully ripened cytoplasm (orthochromatic in the strict sense) are rarely met with in normal bone-marrow

step is to select for detailed examination—still using the 16-mm objective—a highly cellular area of the film where the nucleated cells are well stained and well spread Areas such as these can usually be found towards the tails of films in the vicinity of marrow particles The cells in these cellular areas should be examined first with the 4-mm objective and finally with the oil-immersion 2-mm objective and × 6 eyepieces A look-out should be made at an early stage of the examination for megakaryocytes, which are most often found towards the tail of the film

Systematic examination, backed by a knowledge of the patient's peripheral blood count and his history, will usually enable a diagnosis to be made without recourse to a differential count. A detailed "myelogram" is, in fact, not often required in clinical practice A description of the general cellularity of the marrow, and the type of erythropoiesis and the general maturity of the erythropoietic and leucopoietic cells, and perhaps an estimate of the erythroid-myeloid ratio based on a count of 200 to 500 cells, are all that are usually needed when reporting on bone-marrow films made for diagnostic purposes.

This is not to say that detailed differential counts are never useful

and need never be done. In leukaemia, in agranulocytosis and in myelomatosis, for instance, the proportion of primitive to maturing cells is important for record or scientific purposes, if not for diagnosis On the other hand, time is often much better spent in examining a series of slides than in performing a detailed differential count on the first few hundred cells seen. A wide search may, for instance, in a case of obscure anaemia, settle the diagnosis by revealing isolated groups of metastatic carcinoma cells.

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CHAPTER 8

LABORATORY METHODS USED IN THE INVESTIGATION OF THE HAEMOLYTIC ANAEMIAS

THE laboratory tests used in the investigation of the haemolytic anaemias can be divided into five categories: (*a*) the demonstration *in vitro* of an increase in the sensitivity of the patient's red cells to certain tests for haemolysis; (*b*) tests for abnormal antibodies adsorbed to the red cells of a patient or free in his serum; (*c*) the study of the survival *in vivo* of the patient's red cells or of normal red cells transfused to him; (*d*) tests for sickling of the red cells and for abnormal types of haemoglobin; (*e*) the demonstration of an increased excretion of products of haemoglobin catabolism.

The first four techniques to be described—the measurement of red-cell osmotic fragility, the osmotic fragility test after incubation of blood at 37°C. for 24 hours, the autohaemolysis test and the mechanical-fragility test—are chiefly used in the investigation of the congenital haemolytic anaemias. A short discussion of the significance of each test follows the description of the method.

OSMOTIC FRAGILITY

Principle. The method to be described is based upon that of Parpart and co-workers (43). Hypotonic saline buffered to pH 7·4 is used, and the blood is added to the hypotonic solutions in the proportion of 1 to 100 The test is carried out at room temperature and haemolysis read photoelectrically

Reagents

A stock solution of buffered sodium chloride (A.R.), osmotically equivalent to 10% NaCl, is made as follows: NaCl, 180 g.; Na_2HPO_4, 27·31 g., and $NaH_2PO_4,2H_2O$, 4 86 g are dissolved in distilled water and the final volume adjusted to 2 litres. This solution will keep for months without deterioration in a well stoppered bottle. In preparing hypotonic solutions for use it is convenient to make first a 1% solution from the 10% stock solution, by dilution with distilled water. Dilutions equivalent to 0 85, 0 75, 0·65, 0·60, 0 55, 0·50, 0 45, 0·40, 0·35, 0·30, 0·20 and 0·10% NaCl are convenient test concentrations. Intermediate concentrations such as 0 475% and 0·525% are useful in critical work

It is convenient to make up 50 ml. of each dilution. The solutions

keep well at 4°C for some weeks, but they should be discarded if moulds develop.

Method

Heparinized venous blood or defibrinated blood may be used; [illegible] range of hypotonic solutions. The tubes are allowed to stand at room temperature (20°C.) for at least 30 minutes, then remixed and centrifuged for 5 minutes at 2,000 r.p.m. The amount of haemolysis in each tube is then compared with that in the 100% lysis tube (0 1% NaCl), using a photoelectric colorimeter provided with a yellow-green (Ilford 625) filter. The supernatant from the 0·85% NaCl tube is used as the blank. Usually the supernatants can be poured by decantation into the colorimeter cell. With a good colorimeter as little as 1% lysis can be estimated The depth of the cell should be such that the reading on the colorimeter scale for complete lysis does not exceed 50 (optical density 0 5). If necessary, measured volumes of the supernatants can be diluted with an equal volume of 0·10% NaCl or the blood to saline dilution made 1 to 200 at the start of the test

The blood may be added to the hypotonic solutions by means of a glass-capillary automatic pipette calibrated to deliver 0·05 ml (Fig. 18). With this instrument almost exactly equal amounts of blood can be added to each tube, but skill and practice are needed. Alternatively, straight pipettes calibrated to contain 0 05 ml may be used, but this method, although accurate if a dry pipette is used for each addition, is tedious. A more rapid but far less accurate method is to add one "drop" of blood to each tube

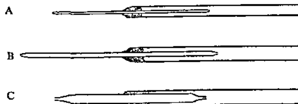


FIG 18 GLASS-CAPILLARY AUTOMATIC PIPETTES

FACTORS AFFECTING OSMOTIC-FRAGILITY TESTS

pH of the blood-saline suspension, and (c) the temperature at which the tests are carried out.

fore, it is recommended that the blood should be mixed until bright red, as occurs during defibrination. Finally, for really accurate work the estimations should always be carried out at the same temperature, though for most purposes "room temperature" is sufficiently constant.

The extent of the effect of pH and temperature on osmotic fragility is illustrated in the paper of Parpart and co-workers (43). The effect of pH is more important, here a shift of 0 1 of a pH unit is equivalent to altering the tonicity by 0 01%, the fragility of the red cells being increased by a fall in pH. A rise in temperature decreases the fragility, a rise of 5°C. being

order to arrest the haemolysis, but this refinement seems unnecessary in practice.

Further details of the factors which affect and control the haemolysis of red cells in hypotonic solutions are given by Ponder (45), Guest (22), and Hendry (25)

Recording the Results of Osmotic-Fragility Tests

Most workers have not been content to record merely the highest concentration of saline at which haemolysis is just detectable ("initial" lysis or minimum resistance) and the highest concentration of saline in which haemolysis appears to be complete ("complete" lysis or maximum resistance) It is useful also to record the concentration of saline causing 50% lysis, i e, the median corpuscular fragility (M.C.F) (54).

When a range of hypotonic solutions has been used a "fragility curve" may be drawn by plotting on graph paper the percentage of haemolysis in each tube against the corresponding concentration of salt solution In normal subjects an almost symmetrical sigmoid curve results (Fig 19) In disease, however, deviations from the normal type of curve are found, e g, curves with long "tails" due to a small proportion of very fragile cells

heterogeneity in the osmotic fragility of the cell population. If the observed amounts of haemolysis of normal blood are plotted on the probability scale against concentrations of saline an almost straight line can be drawn through the points, there being skewness only where haemolysis is becoming almost complete. This method enables the M.C.F. to be read off with ease. In disease, "tailed" curves result in varying degrees of skewness at the other end of the probability plot as well. In order to obtain increment haemolysis curves, the differences in haemolysis between adjacent tubes are plotted against the corresponding saline concentrations, definitely bimodal curves may be obtained during recovery from a haemolytic episode (2, 53).

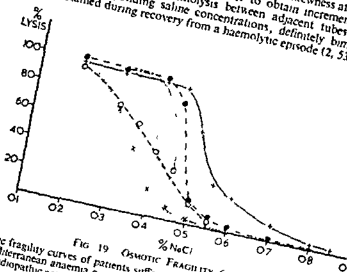


FIG. 19. OSMOTIC FRAGILITY CURVES

Osmotic fragility curves of patients suffering from (a) sickle-cell anaemia × (b) Mediterranean anaemia o - - - - o, (c) hereditary spherocytosis ● — — — ● and (d) idiopathic acquired haemolytic anaemia (warm auto-antibody type) +——+. The shaded area represents the normal range

Normal Range of Osmotic Fragility (at 20° C and pH 7·4)

0·30%	NaCl	97–100%	haemolysis
0·35%	,,	90–99%	,,
0·40%	,,	50–90%	,,
0·45%	,,	5–45%	,,
0·50%	,,	0–5%	,,
0·55%	,,	0%	,,

Median corpuscular fragility (*M.C.F.*) 0·40–0·445% NaCl.

SIGNIFICANCE OF OSMOTIC FRAGILITY TESTS

The osmotic fragility test is probably a measure of spherocytosis, and as spherocytosis may be looked upon as a prehaemolytic change, the test is useful in a patient suspected of suffering from haemolytic anaemia. It provides a more objective measurement of spherocytosis than can be obtained by inspection of stained blood films. It should be emphasized, however, that an increase in osmotic fragility (and the

Osmotic Fragility after Incubation at 37°C. for 24 Hours

Method

Defibrinated blood should be used, care being taken to ensure that the sample is sterile. Duplicate 2-ml. volumes of blood are incubated in sterile 5-ml. screw-capped bottles (It is useful to set up the samples in duplicate, so that in the rare event of a sample being infected, as shown by the haemoglobin being markedly reduced, the whole experiment need not be spoilt.) After 24 hours, the contents of the two bottles are usually pooled after thoroughly mixing the sedimented corpuscles in the overlying serum, and the fragility is then estimated as previously described. As the fragility may be found to be markedly increased, it is advisable to set up additional hypotonic solutions, containing 0 7 and 0·8% NaCl as well as a tube containing 0·85% NaCl. In addition, a solution equivalent to 1·2% NaCl should be used for sometimes, as in hereditary spherocytosis, lysis may take place in 0 85% NaCl, in this case the supernatant of the tube containing 1·2% NaCl can be used as the " blank " in the colorimetric estimation.

The incubation fragility test is conveniently combined with the estimation of the amount of spontaneous autohaemolysis (see later)

Normal Range of Osmotic Fragility after 24 hours at 37°C.:

0·20% NaCl	91–100% haemolysis
0 30% ,,	80–100% ,,
0 35% ,,	72–100% ,,
0 40% ,,	65–100% ,,
0·45% ,,	54– 96% ,,
0 50% ,,	36– 88% ,,
0 55% ,,	5– 70% ,,
0·60% ,,	0– 40% ,,
0 65% ,,	0– 19% ,,
0 70% ,,	0– 9% ,,
0 75% ,,	0– 2% ,,
0 85% ,,	0% ,,

Significance of Osmotic Fragility Tests on Incubated Blood

It has been found that the red cells of patients with hereditary spherocytosis and at least one type of congenital non-spherocytic haemolytic anaemia

undergo, when incubated, a greater increase in osmotic fragility than do normal red cells (49) (Fig 20). It may prove possible, therefore, by the

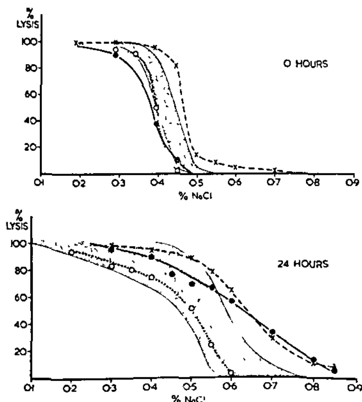


FIG 20 OSMOTIC FRAGILITY CURVES BEFORE AND AFTER INCUBATING THE BLOOD AT 37°C FOR 24 HOURS

From patients suffering from (*a*) hereditary spherocytosis x - - - - x, (*b*) ●———●, and (*c*) o ····· o congenital non-spherocytic haemolytic anaemia The shaded areas represent the normal range

AUTOHAEMOLYSIS (HAEMOLYSIS AFTER INCUBATION OF BLOOD AT 37°C. FOR 24 OR 48 HOURS)

Method

Sterile defibrinated blood is used Four 2-ml samples are delivered into sterile 5-ml screw-capped bottles They are placed in the incubator and left undisturbed for 24 hours The contents of each bottle are then gently mixed by inversion. Two of the bottles are then

replaced at 37°C. and incubated for another 24 hours. The contents of the remaining two bottles are pooled, a sample removed for the estimation of osmotic fragility, a further sample used for the estimation of the packed cell volume (P.C.V.) and the remainder centrifuged to obtain the supernatant serum.

The amount of spontaneous lysis is estimated by means of a photoelectric colorimeter As a rule it is convenient to make 1 in 25 or 1 in 50 dilutions of the incubated serum in 0 04% (v/v) ammonia An appropriate dilution of the pre-incubation serum is used as a blank, and a 1 in 100 or 1 in 200 dilution of the whole blood in 0·04% (v/v) ammonia serves as a standard The percentage haemolysis, allowing for the change in packed cell volume resulting from incubation, is calculated as follows (49):—

$$\text{Percentage haemolysis} = R_T \frac{\frac{100 - P.C.V._T}{100}}{R_o \times 4} \times 100 = R_T \frac{100 - P.C.V._T}{R_o \times 4}$$

R_o = reading in colorimeter of diluted whole blood,
R_T = reading in colorimeter of diluted serum at time T (i.e., at 24 or 48 hours),
$P.C.V._T$ = packed cell volume at time T

Normal Range of Autohaemolysis: Lysis at 24 hours = 0–0·5%.
Lysis at 48 hours = 0 4–3 5%.

Significance of Increased Autohaemolysis

Little or no lysis takes place when normal blood is incubated for 24 hours under sterile conditions, and the amount present after 48 hours is small (49) If glucose is added so that it is present throughout the incubation the development of lysis is markedly slowed In hereditary spherocytosis, however, lysis takes place earlier and progresses more rapidly than normal and the same is true of blood from patients with acquired haemolytic anaemia with spherocytosis and in at least one type of congenital non-spherocytic haemolytic anaemia An accelerated rate of autohaemolysis may also be observed in haemolytic anaemia caused by chemicals such as acetylphenylhydrazine and, most characteristically, in paroxysmal nocturnal haemoglobinuria

[illegible] of normal and abnormal [illegible]

[illegible] haemolysis may thus be due to several [illegible] red-cell [illegible] nenon is [illegible] server is [illegible] he heat-resistance test of Hegglin and Maier (24) is discussed on p 130.

MECHANICAL FRAGILITY

Principle. Red cells are susceptible to mechanical trauma and may be haemolysed readily *in vitro* by shaking with glass beads. In the mechanical fragility test the amount of lysis of the blood under test is compared with that of a normal control blood.

Method

Either defibrinated or heparinized blood may be used. The first step is to adjust the packed cell volume to 45% by withdrawal or addition of serum or plasma, as may be necessary. 2-ml. volumes are then delivered into two 80 × 10 mm. tubes of about 5 ml. capacity. Four glass beads, about 4 mm in diameter, are added to the blood and the tubes closed with tightly fitting rubber bungs. They are then rotated at 33 r.p.m. for 60 minutes at room temperature. At the end of this time the contents of the two tubes are pooled, and 1 to 100 dilutions of the blood are made in 0.04% ammonia and normal saline, respectively. A 1 to 100 dilution in saline of a pre-rotation sample is used as a blank, and the dilution in 0.04% ammonia gives a value for 100% lysis. The amount of lysis is then determined in a photoelectric colorimeter using a yellow-green (Ilford 625) filter.

It is important to set up duplicate samples of a normal blood as a control whenever the mechanical fragility of a patient's blood is being estimated.

Normal range: 2–5% haemolysis

SIGNIFICANCE OF THE MECHANICAL FRAGILITY TEST

[illegible]

The mechanical fragility test, although it provides interesting information, seems hardly likely to be used as a routine laboratory method. The actual technique needs careful standardization and the fact that several different types of red-cell abnormality lead to an increased susceptibility to mechanical trauma reduces its diagnostic value.

SEROLOGICAL METHODS FOR THE INVESTIGATION OF THE ACQUIRED HAEMOLYTIC ANAEMIAS AND HAEMOLYTIC DISEASE OF THE NEWBORN

It is now realized that in most cases of acquired haemolytic anaemia the increased haemolysis is brought about by the development by the patient of auto-antibodies directed against his own red cells. The demonstration of auto-antibodies adsorbed to the patient's red cells or free in his serum is thus of considerable diagnostic importance. Some

of the techniques which will be described below are also applicable to the investigation of haemolytic disease of the newborn.

Auto-antibodies sensitize red cells to antiglobulin serum and often cause them to agglutinate and, less commonly, to haemolyse. Some general points of technique will be dealt with first; next, the direct and indirect antiglobulin tests will be described, and finally tests for agglutinating and haemolytic antibodies.

Collection of Samples of Blood and Serum

The minimum essential requirements are the patient's serum separated from blood allowed to clot at 37°C, and a suspension of freshly taken red cells. If high-titre cold antibodies are suspected, it is advisable, using a needle and short length of rubber tubing, to deliver the patient's blood directly into a container (e g, a 30-ml. screw-capped glass bottle) previously warmed to 37°C. If this is done, unhaemolysed serum can be regularly obtained. The patient's red cells may be obtained from oxalated, citrated or heparinized blood or from blood allowed to clot at 37°C. If cold antibodies, active at room temperature or above, are present, and the direct antiglobulin test is to be carried out, it is well to deliver some blood directly into a large volume of saline warmed to 37°C. and to wash the corpuscles without delay.

When samples are sent by post, it is best to send separately (*a*) serum (separated at 37°C.) and (*b*) whole blood to which sufficient acid-citrate-dextrose (ACD) solution has been added to prevent coagulation.

Storage of Samples

Serum is best stored at −20°C or below in small (1- to 2-ml) volumes Red cells can be kept for as long as 2 to 3 weeks at 4°C. if ACD is used as an anticoagulant, or for longer periods if frozen at −20°C. in citrate-glycerol mixtures (5) They cannot be kept for more than a few hours when washed and suspended in saline

Preparation of Red-Cell Suspensions

Before use, the red cells should be washed in three changes of a large [illegible]

[illegible]

Strength of Red-Cell Suspensions Used in Titrations

0 5 to 1·0% suspensions of red cells in saline are suitable for agglutination tests. For haemolysis tests a stronger (4%) concentration is desirable in order to demonstrate (by colour) minor degrees of haemolysis

Suspensions in Albumin

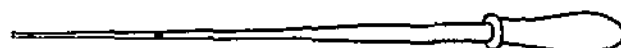


FIG 21 MARKED PASTEUR PIPETTE

Preparation of Serial Dilutions of Patient's Sera

Several methods are available for making serial dilutions of sera·

from " carry over " of serum—that is to say traces of concentrated sera remain

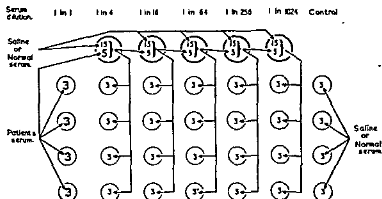


FIG 22 DIAGRAM ILLUSTRATING METHOD OF PREPARING FOUR SETS OF FOURFOLD DILUTIONS OF A SERUM

Fourfold Dilutions Using a Drop Method

The "drop" method is useful when fourfold dilutions of the patient's serum are to be made, a method of dilution which is economical in tubes and is adequate for most purposes

When comparing the effect of different temperatures on agglutination, or the different sensitivities of several types of cells, it is convenient to make

TABLE VIII

COMPARISON OF TITRATION END-POINTS OF A HIGH-TITRE COLD AGGLUTININ USING CONVENTIONAL DOUBLING-DILUTING TECHNIQUES WITH THOSE OBTAINED BY MAKING DILUTIONS WITH SEPARATE PIPETTES

Final serum dilution		1/1,024	1/4,096	1/16,000	1/64,000	1/256,000	1/1,000,000	Control (saline)
1 *Doubling dilutions using Pasteur pipette* (*a*) no mixing in stem	macro	+	+	+	±	0	0	0
	micro	+	+	+	±	±	[±]	0
(*b*) with mixing in stem	macro	+	±	±	trace*	0	0	0
	micro	+	+	+	+	±	[±]	0
2 *Doubling dilutions using glass automatic pipette*	macro	+	+	±	±	(?)	0	0
	micro	+	+	+	±	±	±	0
3 *Dilutions prepared using a separate pipette for each dilution*	macro	+	+	±	(?)	0	0	0
	micro	+	+	±	[±]	0	0	0

* The titre read macroscopically was recorded as 32,000

Only the results of readings made on the last 7 alternate tubes of the titrations are recorded. The doubling dilutions were prepared commencing with undiluted serum, and the separate dilutions (series 3) were prepared from an initial serum dilution of 1 in 256. The titrations were carried out at room temperature (18°C) and the end-points of the titrations were determined macroscopically (macro) using a concave mirror, and also microscopically (micro). The end-points are indicated thus [±].

The end-points determined *macroscopically* using the conventional doubling-diluting techniques give results which closely approximate to the truth, assuming that the correct titration figure is obtained when the dilutions of serum are prepared using a separate pipette for each dilution and the result read microscopically.

the serum dilutions in a series of 75×10-mm. tubes and to deliver by the drop method subsamples of each dilution into the 75×8-mm tubes used for the agglutination tests (Fig 22) The master dilutions can be made by the drop method or if greater accuracy is desired using separate pipettes for each dilution.

The diluent should be saline for agglutination tests or undiluted fresh normal serum compatible with the red cells used for haemolysis tests. The normal serum serves as a source of complement

Size of Tubes

[illegible]

Reading Agglutination and/or Haemolysis

Agglutination

This may be read. macroscopically, as in antiglobulin tests carried out on tiles, microscopically, as in antibody titrations in albumin; or macroscopically, using a concave mirror, as in reading the results of cold-agglutinin titrations In each case the results are scored as follows:

Tile Tests

++++ is the strongest reaction—almost complete agglutination occurring in a matter of seconds, ± is a weak reaction but unquestionably different from the control—it may not be apparent until 5 minutes have elapsed, +, ++ and +++ are intermediate reactions of [illegible]

the cell

ance of

agglutination visible to the naked eye can be recorded (62) This method of reading is useful in comparing the degree of sensitization of several batches of red cells or the avidity of different antiglobulin sera in agglutinating a single sample of sensitized cells.

Tube Tests

When read *macroscopically* with a concave mirror after gently resuspending the deposited cells, ++++ is intense agglutination

which results in a button of cells which remains undispersed when the tube is inverted; a ± reaction is a distinct granularity, persisting after inverting the tube, compared with the control saline suspension; +, ++ and +++ are intermediate degrees of agglutination visible to the naked eye.

A good idea of the presence or absence of agglutination can be obtained by inspection of the deposit of sedimented cells; a perfectly smooth round button suggests no agglutination whilst agglutination is shown by varying degrees of irregularity, " graininess " or dispersion of the deposit. The presence or absence of agglutination, however, must be confirmed microscopically or by inversion of the tubes.

When read *microscopically*, ± agglutination is recorded when uniformly distributed but widely separated small agglutinates (3 to 6 cells) are present in a sea of unagglutinated corpuscles. (For the differences between weak agglutination and rouleaux formation see p. 202.)

The *agglutinin* titre is recorded as the reciprocal of the highest final serum dilution (after allowing for the addition of the corpuscles) in which there is ± agglutination.

Quantitative Measurement of Agglutination

Quantitative methods of reading the results of agglutination tests are desirable in comparing sera or the agglutinability of cells The simplest method is to describe the degree of agglutination in each tube by a figure (47), e.g , +++ = 10, ++ - 8, + = 5, ± or (+) = 3, weak (w) or trace = 2 The figures for each tube are added together to give the titration " score " for the serum The score may then be multiplied by the number of tubes (in a doubling dilution) in which agglutination has occurred The final figure gives a measure of the titre as well as of the intensity of agglutination

The accuracy of the above method is unfortunately not high, for the visual assessment of agglutination is subject to considerable personal error, even if the readings are made by a single observer Nevertheless, the method suffices for most purposes

Two alternative methods of reading agglutination will be mentioned Carlinfanti and Molla (4) placed relatively large volumes of serum dilution and cell suspension in narrow bore tubes so as to give columns about 5 cm. in height The suspensions were allowed to agglutinate and sediment undisturbed for 4 hours. The turbidity of the supernatant sedimenting [illegible] cells, [illegible] then compared with that of a suspension [illegible] s of [illegible] con- [illegible] ches [illegible] cells [illegible] that [illegible]

transformed into probits. The HD_{50}, the haemagglutination dose agglutinating 50% of cells, can be read off a straight line fitted to the probits.

Haemolysis

This is read qualitatively after centrifuging the suspensions and comparing the colour of the supernatant with that of the control. ++++ represents complete haemolysis; ± is definite but weak haemolysis compared with that of the control; + is a pale-red supernatant, and ++ and +++ deep-red supernatants.

The *haemolytic titre* is given by the reciprocal of the highest final serum dilution causing ± haemolysis.

Haemolysis can be read quantitatively, if sufficient supernatant is [illegible]

puscles have been added serve as blanks

Preparation of Antibody-Sensitized Red Cells

[illegible]

Preparation of Cold-Antibody-Absorbed Normal Serum

[illegible]

usually sufficient

DETECTION OF INCOMPLETE ANTIBODIES: THE ANTIGLOBULIN (COOMBS) TEST

Principle of Antiglobulin Test

The antiglobulin test was introduced by Coombs, Mourant and Race (6) in 1945 as a method of detecting "incomplete" Rh antibodies

i.e., antibodies adsorbed by Rh-positive red cells but not capable of

undergo agglutination when subsequently suspended in an anti-human-globulin serum (Coombs's serum) prepared by immunizing a rabbit against human serum (see p. 125).

The same principle had been employed in 1908 by Moreschi (38), who demonstrated that red cells sensitized with heterologous sera could be agglutinated by antibodies formed against the heterologous protein This work, however, had been forgotten. The antiglobulin test has proved to be a sensitive method of detecting the warm and cold auto-antibodies of acquired haemolytic anaemia.

Qualitative Direct Test

The patient's red cells are washed three times in a large volume of saline warmed to 37°C. A 10 to 20% suspension of cells in saline is then made and one drop is mixed on a translucent rectangular tile with a drop of potent antiglobulin serum diluted to the point of maximum avidity (see below). A further drop of the patient's cell suspension is added to a drop of saline as a control. The suspensions are gently rocked from time to time and are viewed with the naked eye or with a hand lens. At the end of 5 minutes, or 7 minutes at the most, the results are read, illuminating the tile from below by means of an electric lamp. As controls, normal unsensitized cells and cells previously weakly sensitized in an anti-D serum should be suspended in the anti-globulin serum; the former suspension acts as a control for the specificity and the latter as a control for the sensitivity of the reaction.

Warm saline is recommended as the washing solution so that if the patient's red cells are auto-agglutinated—as in acquired haemolytic anaemia associated with high-thermal-amplitude cold antibodies—the agglutinin may be washed off and a smooth suspension of cells obtained. There seems to be no risk of washing off incomplete cold antibodies by using saline warmed to 37°C.

Quantitative Direct Test

The qualitative test described above is deficient in two respects: it gives only a rough idea of the strength of sensitization and it assumes (erroneously) that corpuscles sensitized with different types of antibody react equally readily with a single dilution of antiglobulin serum It is preferable, therefore, to use a simple quantitative antiglobulin test in the investigation of suspected cases of acquired haemolytic anaemia.

Method. Serial fourfold dilutions of the antiglobulin serum are made in saline by means of a drop method. One drop of each dilution (usually 1 in 4 to 1 in 4,096) is delivered by means of a fine Pasteur pipette serially on to a large opalescent tile; one drop of saline serves

as a control. One drop of a 10 to 20% red-cell suspension is added to each dilution of the antiglobulin serum and to the saline control. The suspensions are then mixed in succession using the corner of a glass slide, starting with the control and finishing with the highest concentration of the antiglobulin serum. A series of dilutions of the antiglobulin serum are also set out for the control normal cells and control sensitized cells, respectively

The results are read after 5 to 7 minutes, and scored from ++++ to ± (see page 105). Illustrations are given in Table IX and Fig 23.

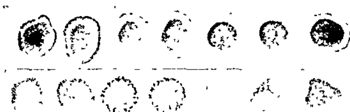


FIG 23 PHOTOGRAPH OF ANTIGLOBULIN REACTIONS CARRIED OUT USING VARIOUS DILUTIONS OF AN ANTOGLOBULIN SERUM (SEE ALSO TABLE IX)

Upper series Red cells sensitized with a normal incomplete cold antibody
Lower series Red cells sensitized with incomplete anti-D

The dilutions of the antiglobulin serum ranged from 1 in 4 to 1 in 4,096 The red-cell suspension on the extreme right is the control, with saline substituted for antiglobulin serum

The tile technique described above is convenient and sensitive, the results are clear-cut and easily read, and agglutination develops quickly. However, the reaction can be carried out in tubes This is the best method when only small volumes of cells are available. For example, when an agglutinin titration has been carried out using a 1% suspension of corpuscles, the cells can be washed in the original tubes after the agglutination has been read and an equal volume of appropriately diluted antiglobulin serum added to each deposit of washed cells After resuspension and incubation for 30 to 60 minutes at 37°C, agglutination can be assessed microscopically, or macroscopically using a concave mirror

Antiglobulin sera vary in their potency, and it must not be assumed that all [illegible]

cells sensitized by warm antibodies are usually agglutinated best in relatively highly diluted antiglobulin sera (prozone type of reaction) (Table IX) cells sensitized by cold antibodies, on the other hand, always seem to be agglutinated most strongly in relatively strong concentrations of antiglobulin serum (Table IX) (13).

TABLE IX

THE EFFECT OF DILUTING AN ANTIGLOBULIN SERUM ON ITS ABILITY TO AGGLUTINATE THE RED CELLS OF PATIENTS SUFFERING FROM ACQUIRED HAEMOLYTIC ANAEMIA

Case number or antibody	Type of antibody (W = warm) (C = cold)	Dilutions of antiglobulin serum: 1 in 4	1 in 16	1 in 64	1 in 256	1 in 1,024	Control (saline)
Anti-D	W	+	+±	++	+	±	0
9	W	++	+++	++++	+++	+±	0
10	W	±	+	++	++	±	0
K_1	W	+±	++	+±	+	±	0
R_1	W	++	++±	++	±	0	0
Anti-H (normal incomplete cold antibody)	C	++	+	trace	0	0	0
13	C	++	+	trace	0	0	0
14	C	+++	+±	trace	0	0	0
18	C	++	+±	±	trace	0	0

Cases 9, 10, K_1 and R_1 were suffering from acquired haemolytic anaemia of the warm-antibody type, and Cases 13 and 14 from acquired haemolytic anaemia of the cold-antibody type. Case 18 was suffering from paroxysmal cold haemoglobinuria. The results with corpuscles sensitized by incomplete anti-D and incomplete anti-H, respectively, are shown for comparison. +++ denotes strong agglutination; ± weak but definite agglutination; +, +±, ++ and ++± denote intermediate grades of agglutination. The optimum dilution of the serum is marked

TABLE X

THE EFFECT OF THE ADDITION OF *HUMAN* γ GLOBULIN TO A RABBIT ANTI-HUMAN *GLOBULIN* SERUM ON THE ABILITY *OF THE* LATTER TO AGGLUTINATE THE *RED CELLS* OF PATIENTS SUFFERING FROM ACQUIRED HAEMOLYTIC ANAEMIA

Case number or antibody	Type of antibody (W = warm) (C = cold)	Dilutions of 4% γ-globulin solution					
		1 in 4	1 in 16	1 in 64	1 in 256	1 in 1,024	Control (saline)
Anti-D	W	0	0	0	0	++	+++
9	W	0	0	0	0	+++	+++
10	W	0	0	0	0	trace	++
K1	W	trace	+	+	+	+±	++±
R1	W	trace	+	+±	++	+++	+++
Anti-H (normal incomplete cold antibody)	C	+	++	+++	+++	+++	+++
13	C	+	+	++	++	++	++
14	C	+	++	++	++	++	++
18	C	++	++	++	++	++	++

Cases 9, 10, K1 and R1 were suffering from acquired haemolytic *anaemia* of the warm-antibody type, and Cases 13 and 14 from acquired haemolytic anaemia of the cold-antibody *type*. Case 18 was suffering from paroxysmal cold haemoglobinuria. The results with corpuscles sensitized by *incomplete anti-D* and incomplete anti-H, respectively, are shown for *comparison*.

+++ denotes strong agglutination, ± weak but *definite* agglutination;

+, +±, ++ and ++± denote *intermediate* grades of agglutination.

THE γ GLOBULIN NEUTRALIZATION TEST

Principle. By
globulin serum,
reaction in acqui
very small conce

haemo

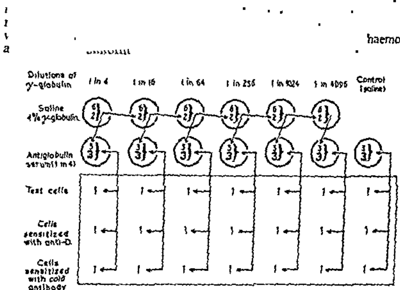


FIG 24 DIAGRAM ILLUSTRATING THE USE OF ANTIGLOBULIN SERUM TO WHICH DILUTIONS OF γ GLOB

Method Fourfold dilutions of a 4% solution of human γ glob
made in saline

so that when the saline is removed after the final washings the packed cells at the bottom of the tubes can be used without any further manipulation)



FIG. 25 PHOTOGRAPH OF ANTIGLOBULIN REACTIONS CARRIED OUT WITH AN ANTIGLOBULIN SERUM TO WHICH VARYING AMOUNTS OF HUMAN γ GLOBULIN HAD BEEN ADDED (SEE ALSO TABLE X)

Upper series Red cells sensitized with a normal incomplete cold antibody
Lower series Red cells sensitized with incomplete anti-D

The dilutions of 4% γ globulin varied from 1 in 4 to 1 in 4,096 The red-cell suspension on the extreme right is the control, with saline substituted for γ globulin

The absorptions should be repeated until the antiglobulin serum, diluted to its point of maximum avidity, no longer agglutinates the cells used to absorb it. With cells sensitized with anti-D, as many as eight absorptions may be needed (using two volumes of cells to one volume of antiglobulin serum (Crawford and Mollison (7)), but with cells sensitized with cold antibodies fewer absorptions will usually prove sufficient

In practice, it is not always necessary to absorb the antiglobulin serum completely in order to determine whether two antibodies are reacting with the same component in the serum For instance, a reaction apparently of the cold-antibody type may be compared with that of known cold-antibody-

marked diminution in the strength of the agglutination by the antiglobulin serum of both types of cells irrespective of the cells used for absorbing the serum

SIGNIFICANCE OF THE DIRECT ANTIGLOBULIN TEST IN THE DIAGNOSIS OF ACQUIRED HAEMOLYTIC ANAEMIA

A positive direct antiglobulin test is a valuable pointer to the presence of auto-antibodies However, a positive test cannot be taken

to be necessarily diagnostic of acquired haemolytic anaemia, nor, on the other hand, does a negative test exclude the diagnosis. The sensitivity of the test creates pitfalls in interpretation. Excluding false positive tests due to the use of inadequately absorbed antiglobulin sera, positive tests may occasionally be given by the blood of patients suffering from a variety of diseases or even by that of a normal subject.

One type of positive reaction is due to sensitization occurring *in vitro*. If, for instance, clotted or defibrinated normal blood is allowed to stand in a refrigerator at 4°C. and the antiglobulin test is subsequently carried out on red cells obtained from the chilled blood, the reaction may be positive due to adsorption of the incomplete cold antibodies normally present in human sera (11). Cells obtained from chilled oxalated or heparinized blood are less likely to give this type of positive reaction as the presence of anticoagulants inhibits sensitization. However, sensitization by cold antibodies is not the only cause of unexpected positive antiglobulin reactions. In some instances, the reaction will be found to be positive even if the possibility of chilling *in vitro* has been excluded by collecting the patient's blood directly into saline warmed to 37°C. The cause of this type of "non-specific" reaction is

reduced, but this does not seem to be true of all. Although abnormal

Falsely negative reactions may be due to three main causes: the antiglobulin serum may be relatively impotent and only capable of detecting strongly sensitized corpuscles; the corpuscles to be tested may have been insufficiently washed free of plasma or serum, and the antiglobulin serum may have been used at an inappropriate dilution.

Unexpected negative reactions are sometimes found in patients who otherwise would seem to be suffering from haemolytic anaemia of the auto-

sera failed to do so, may be quoted as an example ... routine methods of investigation in a particular instance. Whether Evans's observation provides the explanation for other failures remains to be seen.

INDIRECT ANTIGLOBULIN TESTS

The serum of patients suffering from acquired haemolytic anaemia may sometimes be shown to contain free auto-antibodies. However, warm antibodies, detectable by the indirect antiglobulin test, are usually found only in sera from patients suffering from a serious degree of haemolysis. On the other hand, antibodies detectable by enzyme-treated red cells may be found in the sera of most patients, even if they are in clinical and haematological remission (13). If the haemolytic anaemia is of the cold-antibody type, incomplete cold antibodies are usually readily demonstrable by means of the antiglobulin test in the sera of most patients.

The following points must be considered when attempting to detect, by means of the antiglobulin reaction, antibodies in the sera of patients with acquired haemolytic anaemia: the optimum temperature for sensitization; the optimum pH, the necessity for fresh serum, the specificity of the antibodies and the appropriate type(s) of normal red cells that must be used, the concentration of red cells to be added to the serum and the duration of sensitization, and the optimum dilution of the antiglobulin serum.

Optimum Temperature

volume of warm saline, as a preparation for centrifugation, before being removed from the water-bath.) Tests set up at 0° to 4°C usually give less valuable information, as positive results are produced by the incomplete cold antibodies present in normal sera.

Optimum pH

Necessity for Fresh Serum

Cold antibodies fail to sensitize red cells to antiglobulin serum if the serum used for the sensitization has been previously heated at 56°C for 5 minutes or longer. It seems possible that all four fractions of complement are

The adsorption and fixation of warm antibodies composed of γ-globulin is *not* influenced by the presence of complement, and tests can be carried out even on heat-inactivated sera.

Specificity of the Antibodies

Concentration of the Red Cells and the Duration of Sensitization

If supplies of serum permit, it is probably best to use at least 5 drops for each test and to add to the serum 1 drop of a 20 to 30% suspension of washed normal red cells Incubation at 37°C., or the selected temperature, should be prolonged for at least 2 hours. Red cells sensitized by warm

Optimum Dilution of Antiglobulin Serum

As described on page 109, red cells sensitized by warm antibodies are

1 in 4 and 1 in 64

Procedure for the Detection by the Antiglobulin Reaction of Warm or Cold Antibodies in the Sera of Patients with Acquired Haemolytic Anaemia

Based on the considerations outlined in the preceding paragraphs the following suspensions should be set up.

Tube (1), patient's serum 5 vol (5 drops) + 20 to 30% suspension of normal group-O *CDe/cDE* red cells 1 vol. (1 drop) Incubate for 2 hours at 37°C.

Tube (2), as Tube (1), but at 20°C

„ (3), as Tube (1), but with the serum previously acidified with a one-tenth volume of 0 25 N-HCl.

„ (4), as Tube (3), but at 20°C.

„ (5), as Tube (1), but with an equal volume of fresh normal serum added to the patient's serum.

„ (6), as Tube (5), but at 20°C.

Tube (7), as Tube (5), but with the sera acidified with a one-tenth volume of 0·25 N-HCl.

" (8), as Tube (7), but at 20°C.

" (9), as Tube (3), but using patient's serum which has been inactivated at 56°C. for 30 minutes.

" (10), as Tube (9), but at 20°C

" (11), as Tube (1), but with normal serum instead of the patient's serum.

" (12), as Tube (11), but at 20°C

" (13), as Tube (3), but with normal serum instead of the patient's serum.

" (14), as Tube (13), but at 20°C.

All the tubes are allowed to stand at 37°C (or 20°C.) for at least 2 hours, the cells being gently resuspended in the serum from time to ime At the end of 2 hours the tubes are inspected for agglutination or haemolysis. The cells are washed in three changes of saline and agglutination tests then carried out on a translucent tile, as described on page 108, using a potent antiglobulin serum diluted 1 in 4 and 1 in 64

If antibody is detected, the next step is to titrate it and determine, if possible, whether it has a definite specificity (see below)

DETERMINATION OF THE SPECIFICITY OF THE ANTIBODY IN A PATIENT'S SERUM OR IN AN ELUATE OF HIS RED CELLS

[illegible]

TITRATING ANTIBODIES BY THE INDIRECT ANTIGLOBULIN METHOD

Warm Antibodies

Doubling or fourfold dilutions of the patient's serum are made in saline, so as to give serum dilutions ranging from undiluted serum to 1 in 1,024.

To each tube is added an equal volume of a 2% suspension of washed group-O *CDe/cDE* red cells. After 2 hours at 37°C., the tubes are inspected for agglutination (if any) and the cells washed three times in a large volume of saline.

If supplies of the serum are sufficient, it is best to use 75×10-mm. tubes and relatively large (i.e., 0·25-ml.) volumes for the titration. *Sufficient*

[illegible]

Cold Antibodies

Cold antibodies can only be satisfactorily titrated by the antiglobulin method if fresh normal human serum is used as a diluent instead of saline (see Table XI). The normal serum must first be absorbed at 0°C. so as to remove the normal incomplete cold antibodies it probably contains (see

[illegible]

DETECTION AND TITRATION OF INCOMPLETE WARM ANTIBODIES USING ALBUMIN AND SERUM-ALBUMIN MEDIA

Direct Tests. Red cells which have absorbed large amounts of warm

[illegible]

serum an equal volume of a 1 to 2% suspension of normal group-O *CDe/cDE* corpuscles in 20% albumin. The presence or absence of agglutination is read microscopically after the suspension has been incubated at 37°C. for 2 hours. It is essential to set up a suspension of the normal cells in a normal

[illegible]

volume of the normal cells suspended in 20% albumin. The [illegible] incubated for 2 hours at 37°C. and the results read microscopically.

The patient's red cells can be substituted for normal cells and titrations carried out as above (17, 42). However, as the patient's cells are probably already sensitized and thus likely to undergo spontaneous agglutination in the normal serum used as diluent, the interpretation of titration values is open to argument.

Table XI

TITRATION WITH NORMAL RED CELLS OF INCOMPLETE COLD ANTIBODIES DILUTED IN SALINE AND IN NORMAL SERUM, RESPECTIVELY

Serum	Diluent	Serum dilutions					
		1 in 4	1 in 16	1 in 64	1 in 256	1 in 1,024	Control
Normal	Saline	++	trace	0	0	0	0
	Serum	++	+	0	0	0	0
Virus pneumonia	Saline	+++	++	+	0	0	0
	Serum	++++	+++	+	+	+	trace
Idiopathic acquired haemolytic anaemia (Case Ro) (cold-antibody type)	Saline	0	0	0	0	0	0
	Serum	++++	++++	+++	++	++	trace

++++ denotes very strong agglutination by an antiglobulin serum, +++, ++, and + denote lesser degrees of agglutination

The sera were titrated at 4°C

DETECTION AND TITRATION OF INCOMPLETE ANTIBODIES USING ENZYME-TREATED RED CELLS

Enzyme-treated red cells were introduced as reagents for the detection of incomplete antibodies by Morton and Pickles (39, 40) Most workers have used trypsin in the investigation of cases of acquired haemolytic anaemia, but similar results are obtained with other enzymes, such as papain and ficin.

Method. Doubling or fourfold dilutions of the patient's serum are made in saline so as to give serum dilutions ranging from 1 in 1 to 1 in 256 The tubes are placed in the water-bath at 37°C. and equal volumes of a 1% suspension of the enzyme-treated red cells are added to each tube and mixed with the serum dilutions. The deposited cells are resuspended after 1 hour and readings finally made after 2 hours' incubation Agglutination is relatively easy to read macroscopically using a concave mirror and the method seems more sensitive than the serum-albumin method of titration.

Trypsinized red cells not uncommonly react with antibody components which cannot be detected by the indirect antiglobulin test or by the serum-

invariably (27), present in only low concentrations

PREPARATION OF ENZYME-TREATED RED CELLS

Preparation of Trypsin Solution

Crystalline trypsin (Armour) is satisfactory A 1% solution is made by weighing out a few mg of the powder and dissolving in the appropriate volume of 0 05N-HCl. The solution keeps for a week or more at 4°C. A 0·1% solution is then made by diluting one part of the stock solution with nine parts of isotonic pH 7 7 phosphate buffer (1 63% Na_2HPO_4 anhyd., 90 5 parts, 2 34% NaH_2PO_4 $2H_2O$, 9 5 parts)

Trypsinization of Red Cells 0 2 ml of packed washed normal group-O *CDe/cDE* cells is added to 1 ml. of the 0 1% trypsin solution The mixture is incubated for 1 hour at 37°C and the trypsinized corpuscles are then washed in at least two changes of saline

Preparation of Papain Solution (21)

1 g. of papain* is suspended in 100 ml of saline After frequent shaking the suspension is allowed to stand overnight at 4°C. The supernatant is then decanted into small (5-ml) bottles and kept frozen at −20°C. For use, one volume of the papain solution is diluted with nine volumes of buffered saline at pH 7 3 (0 947% Na_2HPO_4, three parts, 0 907% KH_2PO_4, one part. One part of the phosphate buffer is added to nine parts of normal saline)

* Obtainable from Biddle and Sawyer Ltd, 4 Grafton Street, London W 1

Preparation of Ficin Solution*

25 mg of Ficin (Merck) are dissolved in 2 5 ml. of iso-osmotic phosphate buffer at pH 7·3 to 7 5 (2 34% $NaH_2PO_4.2H_2O$, 20 parts, 1·63% Na_2HPO_4, 80 parts). The solution is stable for at least 2 weeks when stored at 4°C If frozen, it probably retains its potency for long periods

Ficinization of Red Cells Red cells are well washed in three changes of saline and a 2% saline suspension made To nine parts of this suspension are added one part of the 1% ficin solution After the mixture has been allowed to stand for 15 minutes at 37°C, the red cells are re-washed in at least two changes of saline The enzyme-treated cells can be stored at 4°C for up to 12 hours before use

DETECTION AND TITRATION OF IN-SALINE-AGGLUTINATING (COMPLETE) ANTIBODIES

Agglutinating warm antibodies are rarely demonstrable in the sera of patients with acquired haemolytic anaemia, they are, however, not unknown (13) Cold antibodies, on the other hand, exist typically as agglutinins

Warm Agglutinins

These can be titrated by making doubling or fourfold dilutions of the patient's serum in saline and adding to each tube an equal volume of a 1% suspension of washed group-O *CDe/cDE* red cells Agglutination is read macroscopically, with the aid of a concave mirror, or microscopically, after incubation for 2 hours at 37°C

Cold Agglutinins

Doubling or fourfold dilutions are made in saline to give serum dilutions ranging from 1 in 1 to 1 in 2,048 (or higher) Equal volumes of a 1% suspension of normal group-O corpuscles are added to each tube.

The suspensions are allowed to stand for 2 hours at 20°C (room temperature) and the agglutination then read macroscopically with the aid of a concave mirror after gently inverting the tubes two or three times to resuspend the sedimented corpuscles (see p. 105) The suspensions are then remixed and the rack of tubes placed in the

* The solution is intensely poisonous If accidentally swallowed, it causes serious necrosis of mucous membranes

refrigerator at 4°C. After chilling for at least 2 hours the agglutinin titres are re-read as quickly as possible before the tubes have had time to warm up appreciably. Finally, the rack of tubes is placed in the water-bath at 37°C., and the cells resuspended. They are examined for agglutination after a further 1 hour's incubation.

Normal corpuscles vary considerably in their agglutinability by the cold antibodies of acquired haemolytic anaemia, even though this variation in sensitivity—which may be more than fourfold—does not seem to be accountable by the presence or absence of known blood-group antigens. It is preferable, therefore, to use blood from the same normal donor whenever red cells are required for cold-agglutinin titrations.

DETECTION AND TITRATION OF HAEMOLYTIC ANTIBODIES

Warm Haemolysins

Antibodies capable of bringing about the haemolysis of normal red cells have seldom been detected in the sera of patients with acquired haemolytic anaemia (13). Haemolytic factors causing the lysis of trypsinized or paroxysmal nocturnal haemoglobinuria (P.N.H.) red cells are met with more frequently

Method. The haemolysins may be titrated by making doubling or fourfold

Cold Haemolysins (Excluding the Donath-Landsteiner Antibody)

Sera containing cold antibodies at high titres can nearly always be shown to be potentially haemolytic. Nevertheless, normal corpuscles are as a rule only haemolysed in acidified sera (10, 14).

suspension comes into contact with the side of the tube, this by itself may lead to haemolysis.

Titration of Cold Haemolysins. Haemolytic high-titre cold antibodies may be titrated by making doubling or fourfold dilutions of the patient's serum in acidified fresh normal human serum (containing 10% by volume of 0·25 N-HCl), and adding to the serum dilutions equal volumes of a 4%

[illegible]

corpuscles are very strongly agglutinated and less strongly haemolysed by high-titre cold antibodies. The haemolysin titre at 20 C., nevertheless, considerably exceeds that of normal red cells, and no acidification of the serum is necessary to demonstrate haemolysis P.N.H. corpuscles are remarkably sensitive to haemolysis and the haemolysin titre at 20 C. is often as great, or may even exceed, the agglutinin titre as measured with normal red cells (13).

[illegible]

DETECTION AND TITRATION OF THE DONATH-LANDSTEINER HAEMOLYSIN

The Donath-Landsteiner antibody of paroxysmal cold haemoglobinuria differs from the haemolytic cold antibodies referred to previously in that it is relatively far more lytic towards normal cells. Moreover, haemolysis always seems to occur in unacidified serum The antibody *causes agglutination and sensitization to antiglobulin serum to about* the same titre as it causes haemolysis

Qualitative Donath-Landsteiner Test

Samples of the patient's blood are delivered directly into two tubes or " bijou " bottles previously warmed in the 37°C water-bath One sample is left to clot at 37°C , the other is placed immediately in crushed ice at 0°C., and left undisturbed for 30 minutes The tube or bottle is then replaced in the water-bath at 37°C without disturbing the clot. The samples are re-examined when the clots have retracted In paroxysmal cold haemoglobinuria the serum expressed by the clot which was chilled before it was warmed should be tinged deeply red with haemoglobin, the serum of the sample kept at 37°C should be entirely free from haemoglobin

Indirect Donath-Landsteiner Test

Serum from the patient is obtained from blood allowed to clot undisturbed at 37°C One volume of a 50% suspension of washed normal group-O red cells is added to nine volumes of patient's unacidified serum. The suspension is chilled in crushed ice at 0°C. for 30 minutes, then placed in the water-bath at 37°C. The tube is centrifuged after 60 minutes at 37°C

Haemolysis visible to the naked eye indicates a positive test. some cases haemolysis occurs within [illegible] te or so of warming.

additional tube containing patient's serum diluted with an equal volume of normal serum should be subjected to the same procedure to allow for the possibility that the patient's serum is deficient in complement. A further control tube, kept strictly at 37°C throughout, should show no haemolysis.

Titration of the Donath-Landsteiner Antibody

Doubling or fourfold dilutions of the patient's serum are made in fresh normal human serum. An equal volume of a 4% suspension of washed group-O corpuscles is added to each tube and the tubes then immersed in crushed ice at 0°C. After 30 minutes the tubes are placed in the water-bath at 37°C. and the deposited cells resuspended. They are centrifuged after 1 hour's incubation and inspected for haemolysis.

ELUTION OF ANTIBODIES FROM SENSITIZED RED CELLS

The preparation of potent antibody-containing eluates from the red cells of patients with acquired haemolytic anaemia is an essential step in the investigation of the specificity of the antibodies. Eluates are probably best made from red-cell stromata. Kidd's (29) acid-elution and Mitchell's (35) techniques both yield potent eluates of warm antibodies.

Kidd's (29) Method

... 20 ml. or more

...

N-HCl, using bromphenol blue as an external indicator

Acid-elution is continued for 10 to 15 minutes at room temperature, gently ... suspension is then

...

fugation and the supernatant, usually a slightly brown clear solution, ... contain the eluted antibodies

Mitchell's Method (35)

Mitchell (35) studied the relationship between temperature and the opt... pH for elution. He found that eluates as satisfactory as those ma... ind... 37°...

vations suggest that the antibodies cannot all be equally successfully eluted by a single standardized procedure.

Principle. Red-cell stroma is precipitated by dialysis of haemolysed washed red cells against a phosphate buffer of low ionic strength. Elution is carried out by heating the stroma at 56°C in an iso-osmotic phosphate buffer at pH 5·7. (For formulae of buffers, see p 220.)

Method. The red cells of the patient are thoroughly washed in several changes of saline. An equal volume of distilled water is then added and the partially lysed red cells are repeatedly frozen at −20°C and rapidly thawed. They may be stored at −20°C for weeks or even months before elution of antibody is attempted.

The lysed red cells are transferred to a collodion sac and dialysed overnight at 4°C, against a large volume of phosphate buffer of ionic strength (*I*) 0·02 at pH 5·4. The precipitated stroma is transferred to a centrifuge tube and washed several times in the buffer used for the dialysis in order to remove as much haemoglobin as possible. A volume of eluting buffer (*I*) 0·15 at pH 5·7, equal to the volume of red cells from which the stroma was derived, is added to the deposit of stroma and the suspension of stroma in buffer heated at 56°C. for 60 minutes. The suspension is then rapidly centrifuged or, preferably, filtered through a funnel warmed to 56°C. using a Green's 904 filter paper. The supernatant which should contain eluted antibodies, is preserved. This is dialysed once more against an iso-osmotic phosphate buffer at pH 7·4. The final solution should be kept at −20°C until it is tested. If the yield of eluted antibody is disappointing, it is worth while repeating the dialysis using buffers of greater ionic strength i.e. (*I*) 0·04 and 0·06.

PREPARATION OF ANTIGLOBULIN SERUM

Several methods may be used and as only a minority of rabbits produce potent sera, it is advisable to immunize several animals at the same time. Full details are given by Mourant (41).

The primary stimulation may be carried out by the alum-precipitated-serum method of Proom (46) or by Slavin's (52) method, using calcium alginate.

Proom's Method

25 ml. of human group-O serum are diluted with 80 ml of distilled water and 90 ml of 10% potash alum added. The pH is adjusted carefully to 6·5 with 5N-NaOH, using bromthymol blue as external indicator. The preparation is centrifuged and the precipitated protein washed twice with saline containing 1 in 10,000 Merthiolate. The volume of the precipitate is then made up to 100 ml with Merthiolate-saline. 5 ml. of this material are injected intraperitoneally into a rabbit, or intramuscularly into each thigh, and the injections repeated 14 days later.

Hyperimmunization

Irrespective of the method of primary immunization, it is advisable to hyperimmunize the rabbits by injecting whole human serum intraperitoneally, then intravenously (61). At least 4

primary inoculation 0·5 ml. of human group-O serum is injected intraperitoneally. The following day 0·1 or 0·2 ml. of serum, diluted in 1 or 2 ml. of saline, is slowly injected intravenously. The rabbit should be bled 5 to 10 days later. The intraperitoneal and intravenous inoculations should be repeated at intervals of 4 to 6 weeks.

Rabbits can usually be bled satisfactorily from their ear veins. The blood is placed at 37°C. so that the clot may retract. The separated serum is then freed from suspended cells by centrifugation, and inactivated by heating at 56°C. for 30 minutes.

Absorption of Serum

The serum is then absorbed with human red cells which have been washed with at least six washings in a large volume of saline. Two absorptions should be carried out: first, with an equal volume of packed group-O cells, and secondly, with group-A_1B cells or with a mixture of A_1 cells and B cells. The cell-serum suspensions should be left for at least 1 hour at 4°C before centrifuging.

The absorbed rabbit serum must be tested for its specificity and potency When diluted 1 in 4 it should not agglutinate washed normal human red cells and it should be capable of agglutinating red cells deliberately weakly sensitized with antibodies such as anti-D or the incomplete cold antibody present in normal sera. It should be titrated with both types of antibody and optimum dilutions determined for each. If the serum is shown to be both sensitive and properly absorbed, it may be brought into use It should be stored frozen at −20°C. in volumes not exceeding 2 ml It keeps well frozen, but rapidly loses potency if kept at 4°C. when diluted in saline.

An alternative method of treatment of the immune rabbit serum which [illegible] has been a[illegible]pted by Meno- [illegible] 10, [illegible] roy [illegible] the [illegible]

TITRATION OF SERUM COMPLEMENT

Doubling dilutions of freshly obtained sera are made in saline so as to give serum dilutions ranging from 1 in 8 to 1 in 128 0·5-ml volumes are [illegible] tubes [illegible] ded an [illegible] l with [illegible] cells [illegible] D. of [illegible] erum- [illegible] l then cell suspensions are placed in the 37°C. water bath [illegible] centrifuged [illegible] from the two con- [illegible]

(4) If a warm antibody is present, what is its specificity?
Test with a panel of cells of known genotype and carry out absorption experiments.

(4a) Are haemolytic components present?
Titrate at 37°C. with N.T and P.N.H. cells, using normal serum as diluent.

(5) Are there any other serological abnormalities?
Estimation of serum complement.
Estimation of serum proteins; separation by paper electrophoresis
Titration of heterophile (anti-sheep) antibodies. Agglutination and complement-fixation tests for anti-viral antibodies and antibodies against Streptococcus M.G.

N cells = normal group-O red cells, *T.N. cells* — trypsinized normal group-O red cells; *P.N.H cells* = paroxysmal nocturnal haemoglobinuria red cells; *D-L* = Donath-Landsteiner

DIAGNOSIS OF PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA (P.N.H.)

Paroxysmal nocturnal haemoglobinuria is a rare but remarkabl[e] disorder, apparently acquired, in which the patient's red cells ar[e] abnormally sensitive to haemolysis by normal constituents of plasma (13, 44) The disease can be simply and reliably diagnosed by means of the acid-serum (Ham's (23)) test

Acid-Serum Test

Principle The patient's red cells are exposed at 37 C to the action of normal or the patient's own serum suitably acidified to the optimum pH for haemolysis (pH 6 5 to 7 0)

The patient's red cells (P N.H red cells) can be obtained fro[m] defibrinated, heparinized, oxalated or citrated blood, and the test ca[n] be satisfactorily carried out even on cells which have been stored fo[r] several weeks in acid-citrate-dextrose solution at 4°C The patient's serum is best obtained by defibrination, for if obtained from blood allowed to clot in the ordinary way at 37°C or at room temperature it will almost certainly be found to be markedly haemolysed. Normal serum can be obtained either by defibrination or from blood allowed to clot spontaneously at room temperature or at 37°C. It is ad[illegible] to use a sample of normal serum [illegible] the haemolysis of P N H [illegible] fresh. Their haemolytic [illegible] if frozen at −20°C [illegible] is stored, even

Method 0·5-ml samples of fresh normal serum and/or patient's serum are acidified by the addition of one-tenth volumes (0 05 ml.) of 0·2 N-HCl After careful mixing, the acidified serum is placed in the water-bath at 37°C and 0 05 ml (or one large " drop ") of a 50%

suspension of patient's washed red cells is added to each tube. The tubes are centrifuged after incubation at 37°C. for 1 hour. In paroxysmal nocturnal haemoglobinuria the cells in the acidified serum will have undergone definite, although incomplete, lysis. No lysis, or at the most a trace of lysis, should be visible in the unacidified sample As essential controls, additional tubes of unacidified and acidified serum, respectively, should be set up and normal red cells instead of patient's cells added to them No lysis should be visible in either tube

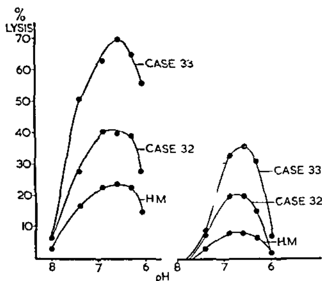


FIG 26 THE EFFECT OF pH ON THE HAEMOLYSIS *in vitro* OF P N H RED CELLS BY NORMAL HUMAN SERA

The red cells of three patients (Cases 32, 33 and H M), of different sensitivity, were used and two fresh normal sera, one serum being more potent than the other

Significance of the Test

The acid-serum test, carried out with proper controls, is positive only in

Other Tests for Paroxysmal Nocturnal Haemoglobinuria

The "heat resistance test" of Hegglin and Maier (24) in which clotted

with acquired haemolytic anaemia and very marked spherocytosis may undergo equally rapid haemolysis (13). P.N.H. red cells are also remarkably

added to it. He proposed that this reaction might also be used as a specific test for the disease.

THE ESTIMATION OF THE LIFE-SPAN OF RED CELLS *IN VIVO*

The Differential Agglutination Method of Ashby

Principle

The recipient is transfused with red cells of a different but compatible blood group; e.g., group-O red cells may be transfused to a group-A or -B or -AB recipient, group-ON cells to a group-OM or -OMN recipient, and Rh-negative cells to an Rh-positive recipient. The resulting mixture of cells may be separated *in vitro* if they are suspended in a potent agglutinating serum in which the recipient's red cells are agglutinated but not those of the donor. For example, when group-O blood has been transfused to a group-A recipient, the group-A cells can be agglutinated by an anti-A serum, but the donor's group-O cells remain in a dispersed suspension Similarly, when group-ON blood is transfused to a group-OMN recipient, the recipient's cells can be agglutinated by an anti-M serum, leaving the donor's group-ON cells unagglutinated.

It is possible to carry out the differential agglutination in a quantitative way and to count the unagglutinated cells with an accuracy hardly less than that for ordinary red-cell counts. However, it is absolutely essential to use a highly avid agglutinating serum. This usually restricts the method to tracing the survival of group-O blood given to a group-A or -B, or -AB recipient, using an anti-A or anti-B serum, or of group-ON blood given to a group-OMN or -OM recipient, using powdered anti-M serum (Lederle) Full details of the use of the differential agglutination method are given by Mollison (36) The technique now to be described is slightly modified from that described by Dacie and Mollison (16)

Method

0 1 ml of venous blood is added to 4 9 ml. of 3% sodium citrate solution to make a 1 in 50 dilution. One volume of the suspension is then added to one volume of the appropriate agglutinating serum

in a 75 × 10 mm. tube provided with a well fitting rubber bung; 0 25 ml. volumes are suitable.

It is good practice to do the test in duplicate if sufficient serum is available The serum should be used undiluted, or diluted in several volumes of saline, if necessary, to the concentration at which agglutina-

used, the best results will be obtained if there are less than 10,000 free cells per c mm, when blood containing 5,000,000 red cells per c mm. is agglutinated by the technique to be described

The dilution of blood in citrate and serum (now 1 in 100) is left at room temperature for at least 2 hours, and then centrifuged at about 1,500 r p m. (about 300 g) for 1 minute The tubes are then quite vigorously shaken so that not only are the unagglutinated cells resuspended but the button of agglutinated cells becomes broken up into small but still visible fragments. After waiting for not more than 1 minute, during which time the largest clumps of agglutinated cells sink to the bottom of the tube, the upper three-quarters of the suspension, consisting of free cells and small clumps only, is removed by a Pasteur pipette into a fresh tube This tube is corked and the contents centrifuged for 1 minute as before The button of deposited cells is then well mixed with the supernatant fluid by a standard procedure—50 inversions through an angle of 90 to 120 at the rate of one per second. A counting chamber is then filled from the upper layers of the cell suspension, thus minimizing the number of agglutinates withdrawn.

After waiting for at least 2 minutes for the cells to settle, a red-cell count is performed in the usual way, counting " free " cells only The cells (usually tightly agglutinated) in the few clumps which may be seen are ignored The number of unagglutinable (donor) cells present may be expressed in absolute numbers or as a percentage of the number present at the conclusion of the transfusion

If anti-M powder is used instead of a liquid agglutinating serum, the powder itself may be added by means of a small wooden spatula (toothpick) to a 1 in 50 or 1 in 100 dilution of the patient's whole blood in 3

per c.mm

The Radio-Chromium (^{51}Cr) Method

Sodium chromate containing a small amount of ^{51}Cr has recently been used in the study of the life-span of red cells *in vivo*. The red cells of one person can be transfused, after tagging *in vitro* with the chromium, to another recipient, or re-injected into the donor. Only

a small volume of blood (less than 20 ml.) need be tagged, and the method has the added advantage over the Ashby method that the life-span of the patient's own cells can be studied in their normal environment. The risk of transmitting serum hepatitis to the recipient is also eliminated in auto-transfusions.

The chromium is thought [illegible]
to impair [illegible]
of the chr[illegible]
in estimating red-cell survival. Full details of the technique and a normal range for the survival of ^{51}Cr-treated red cells is given by Mollison and Veall (37) and Donohue and co-workers (18).

DEMONSTRATION OF SICKLING

The sickling phenomenon may be simply demonstrated by sealing a thin " wet " film of the patient's blood between slide and cover-glass by means of a petroleum jelly and paraffin wax mixture, and then incubating the preparation at 37°C. Sickling develops in the various types of sickle-cell anaemia and also in the sickle-cell trait as the oxygen in the blood is gradually used up. In sickle-cell anaemia well marked sickling is usually visible after incubation for an hour or less at 37°C.; filamentous forms are conspicuous. In the trait the process is slower and the sickling change less severe, " holly-leaf " forms being characteristic. Up to 12 hours' incubation may be necessary for the changes to develop. They can, however, be hastened by the addition of reducing agents to the blood. The method recommended by Itano and Pauling (28) is a reliable one.

Method Using a Reducing Agent

Required *A* 0·114M-$Na_2S_2O_4$ (sodium dithionite)
B 0·114M-Na_2HPO_4 (disodium hydrogen phosphate)

The two reagents are mixed together to give a final pH of about 6·8, about 2 volumes of *A* to 3 volumes of *B* are required.

The sodium dithionite solution should be made up on the day it is required and the appropriate amount of sodium phosphate solution added just before use.

About 0·05 ml. of the reagent is added to a very small drop of blood (0·01 ml.) on a slide and the mixture immediately covered with a cover-glass and sealed with petroleum jelly-paraffin wax mixture.

PHYSICO-CHEMICAL METHODS USEFUL IN THE INVESTIGATION OF ABNORMAL HAEMOGLOBINS

Brief accounts will be given (*a*) of a method for the determination of the rate of denaturation by alkali, and (*b*) of a method of paper electrophoresis applicable to the differentiation of the human haemo-

globins The first stage of either method is the preparation of concentrated stroma-free haemoglobin solutions.

Preparing the Haemoglobin Solutions

[illegible]

METHOD FOR THE QUANTITATIVE MEASUREMENT OF THE RATE OF ALKALI DENATURATION

Principle The alkaline haematin is estimated by measuring the increasing optical density of a solution of haemoglobin in red (640 mμ.) light after the addition of alkali As the absorption due to alkaline haematin at 600 to 650 mμ. is much greater than that of oxyhaemoglobin, the development of the alkaline haematin can be accurately recorded.

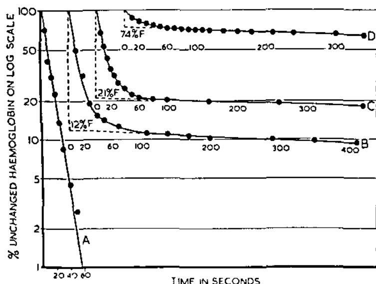


FIG 27 DENATURATION RATES OF HAEMOGLOBIN BY ALKALI

A Sickle-cell trait No foetal haemoglobin present (a normal result)
B Mediterranean anaemia (thalassaemia major) 12% foetal haemoglobin present
C Sickle-cell anaemia (child aged 1 year) 21% foetal haemoglobin present
D Cord blood from normal full-term infant. 74% foetal haemoglobin present (from White and Beaven (59))

Method (57)

are divided by the total density increment and the resultant values plotted on the log-axis of semi-log paper against time, a linear plot, representing

Filter-Paper Electrophoresis of Human Haemoglobins

Principle. Small differences in the iso-electric points of the various human haemoglobins allow their separation by electrophoresis. The differences in behaviour between adult and foetal haemoglobin is slight, but sickle-cell haemoglobin and haemoglobin-D move more slowly towards the anode than normal haemoglobin on paper electrophoresis at the alkaline side of the iso-electric points. Haemoglobin-C has even less anodic mobility than haemoglobins-S and -D under these conditions, and that of haemoglobin-E lies between haemoglobin-S and haemoglobin-C The mobility of haemoglobin-G is intermediate between that of haemoglobin-F and haemoglobin-S, whilst that of the recently studied haemoglobin-H is greater even than that of haemoglobin-A (51, 59) (Table XII).

Method

Haemoglobin Solution

Although oxyhaemoglobin, methaemoglobin and carboxyhaemoglobin behave similarly on paper electrophoresis, the carboxy compound is preferable as it is very stable and readily resolved Solutions of carboxy-

concen-
monoxide
hrough
l out as

a film.

TABLE XII

DIFFERENTIATION OF THE HUMAN HAEMOGLOBINS

Type of haemoglobin	Electrophoretic mobility on filter paper (pH 8 6) (Compared with A haemoglobin)	Resistance to alkali denaturation	Solubility of reduced haemoglobin (Compared with A haemoglobin)	Association with sickling
A (normal)	—	Not resistant	—	No sickling
F (foetal)	Slightly slower than Hb-A	Resistant	Slightly greater than Hb-A	" "
S (sickle)	Slower than Hb-A	Not resistant	Much less than Hb-A	Sickling
C	Slower than Hb-S (the slowest of all)	" "	Greater than Hb-A	No sickling
D	Same as Hb-S		Close to Hb-A	" "
E	Slower than Hb-S, but faster than Hb-C	" "	(?) As Hb-A	" "
G	Slower than Hb-F, but faster than Hb-S	" "	(?)	" "
H	Faster than Hb-A	(?)	(?)	" "

Apparatus

Paper strips ...

... box enclosing the frame and paper, and an air-tight lid is provided (Fig. 28)

Buffer. Barbitone buffer at pH 8·6 of ionic strength (I) = 0 05

Barbitone sodium	..	5 g
Hydrated sodium acetate	..	3 33 g.
0·1 N-sulphuric acid	..	34·2 ml.
Hydrated copper sulphate	..	1 mg
Water to	..	1 litre

Potential and Current. A potential of 100 to 240 volts is applied across the paper, the current being 5 to 10 milliamps. Dry-cells or a stabilized mains D C. supply are suitable.

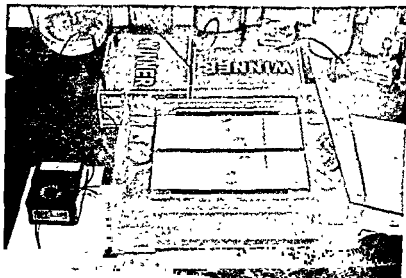


FIG 28 APPARATUS FOR ELECTROPHORESIS OF HAEMOGLOBIN SOLUTIONS

The apparatus ... e show ... 00 volt ... at the ... by

J C White, G H Beaven and M Ellis in *CIBA Foundation Symposium on Paper Electrophoresis* (1956) Churchill, London

Setting Up the Test

Lines are drawn with pencil across each paper strip, between the centre of the paper and the ends ... paper, ... the ... ng ... nd

equilibration of buffer over the paper allowed to take place for 1 hour.

mixture, etc., the other is the unknown solution under test. Inclusion of such markers greatly facilitates precise identification of unknown specimens, as conditions for observation of absolute mobilities are difficult to achieve in paper electrophoresis.

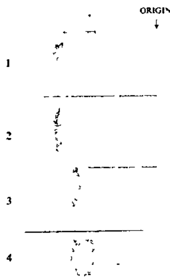


FIG 29 RESULTS OF PAPER-STRIP ELECTROPHORESIS OF HUMAN HAEMOGLOBINS AT pH 8 6

The arrow marks the place of origin (1)=normal (A) haemoglobin, (2)=sickle-cell trait (haemoglobin-A plus haemoglobin-S), (3)=sickle-cell anaemia (haemoglobin-S alone), (4)=haemoglobin-S–C disease (haemoglobin-S plus haemoglobin-C)

Examination of Results

At the end of the separation, the strips are removed and hung up to dry in

and the background very light. They are then dried and rendered translucent in a mixture of liquid paraffin and 1-bromonaphthalene

A direct recording of the density along the prepared paper strips can be made by an automatically-recording densitometer (31).

The relative proportions of the components into which a mixture has been resolved can be determined by measuring the area under each peak in the density tracing by means of a planimeter.

EVIDENCE OF INCREASED HAEMOGLOBIN CATABOLISM

Methods for the estimation of plasma bilirubin, plasma haemoglobin and faecal and urinary urobilinogen and for the detection of urinary haemosiderin will be described. The spectroscopic demonstration of methaemalbumin in plasma is described on p. 215.

Estimation of Serum Bilirubin

Principle

Serum (or plasma) is treated with diazotized sulphanilic acid, with the addition of ammonium sulphate and alcohol to precipitate protein. The red colour produced is compared in a photoelectric colorimeter with that of an artificial standard (2 9 mg. methyl red per litre at pH 4·63). The colour of this solution accurately matches the colour obtained when 0 04 mg of bilirubin is treated with the diazo reagent in a final volume of 10 ml

Method (30)

1 ml. of plasma or serum is treated in a centrifuge tube (or, better, in a glass-stoppered tube) with 0 5 ml of diazo reagent If the diazo reagent is carefully "layered" above the plasma, and the tube allowed to stand for a few moments, a positive "direct" van den Bergh reaction (if present) may be seen at the liquid junction 0 5 ml of saturated ammonium sulphate and 8 ml of 85% (v/v) ethanol are added The mixture is stoppered, thoroughly mixed, allowed to lie on its side for 30 minutes, and then filtered Under these conditions the dilution of the plasma closely approximates 1 in 10

The colour of the clear filtrate is compared with the standard mentioned above (≡ 0 04 mg of bilirubin in a volume of 10 ml), using a green filter (Ilford 624).

If the concentration of azobilirubin in the test appears to be more than twice that in the standard, a suitable dilution of the original plasma with a phosphate buffer solution (see below) should be made, and the procedure repeated. Since this involves dilution of the plasma (e g, 1 in 3 or 1 in 10), the resultant reading must be multiplied by the dilution factor. Obviously icteric plasma should be diluted in the first instance.

Calculation

Photoelectric Colorimeter

$$\text{Bilirubin (mg per 100 ml)} = \frac{\text{Reading of test}}{\text{Reading of standard}} \times 0\ 04 \times \frac{100}{1}$$

$$= \frac{\text{Reading of test}}{\text{Reading of standard}} \times 4$$

Solutions

Stock Standard Methyl-Red Solution 290 mg. of pure methyl red are

Diazo Reagent. This is made by mixing two solutions, *A* and *B*

Solution A is made by dissolving 1 g of sulphanilic acid in 250 ml. of N-hydrochloric acid, and making the volume up to 1 litre with water.

ml of water.

Ammonium Sulphate Saturated solution

SIGNIFICANCE OF INCREASED SERUM-BILIRUBIN CONCENTRATIONS

In the haemolytic anaemias the serum-bilirubin concentration usually lies between 1 to 3 mg per 100 ml Sometimes, however, the concentration may remain within the normal range, despite a considerable increase in the rate of haemolysis The direct van den Bergh reaction is usually negative Serum concentrations exceeding 5 mg per 100 ml and/or the presence of direct-reacting pigment suggest the presence of liver disease.

ESTIMATION OF PLASMA HAEMOGLOBIN

The method described below is a modification of that of Bing and Baker (1)

Principle

must be scrupulously clean

Method

0 1 ml of plasma (or a larger volume of an appropriate dilution of the plasma, see later) is added to 2 ml of the benzidine reagent and 1 ml of the hydrogen peroxide solution in a large test tube A control tube, in which 0 1 ml of distilled water is substituted for the plasma, and a standard tube,

using the colour developed by the control tube as a blank. A blue-green (Ilford 624) filter is suitable.

If the haemoglobin content of the plasma to be tested is abnormally high, the plasma should be diluted until it is just visibly tinged with haemoglobin.

Reagents

Benzidine Solution. 0 5 g of pure benzidine dihydrochloride (Merck) is dissolved in 15 ml. of hot (not boiling) distilled water, and then 25 ml of 95% (v/v) ethanol and 10 ml. of glacial acetic acid are added. The benzidine solution will keep for several weeks in a dark bottle at 4°C.

Hydrogen Peroxide. 0·6% (v/v) solution, prepared by diluting a 3% ("10 vols") solution with distilled water before use.

Normal Range

1 to 4 mg. haemoglobin per 100 ml. plasma (8).

Significance of Raised Plasma-Haemoglobin Concentrations

The plasma-haemoglobin concentration is raised in haemolytic anaemias in which haemolysis occurs predominantly in the blood stream (intravascular haemolysis). Thus marked haemoglobinaemia, with or without haemoglobinuria, is typically found in paroxysmal nocturnal haemoglobinuria, cold haemoglobinuria, blackwater fever, etc Lesser amounts of haemo-

concentrations are normal. It must be emphasized that the presence of excess haemoglobin in the plasma is a reliable sign of intravascular haemolysis only if the observer can be sure that the lysis has not been caused during or after the withdrawal of the blood

ESTIMATION OF UROBILINOGEN IN FAECES

Principle

The stercobilin pigments of the faeces are reduced to urobilinogen, which is extracted with water. The solution is then treated with Ehrlich's dimethylaminobenzaldehyde reagent to produce a pink colour which can be compared with either a natural or an artificial standard.

Method (30)

carbonate solution, diluted to the mark with water and mixed

[illegible]

that of the test.

Calculation

Urobilinogen (mg. per 100 g faeces)

$$= \frac{\text{Reading of test—Reading of blank}}{\text{Reading of standard}} \times 0.00387 \times V^{*} \times \frac{100}{0.1}$$

$$= \frac{\text{Reading of test—Reading of blank}}{\text{Reading of standard}} \times V^{*} \times 3{\cdot}87$$

* Final volume of the coloured solution (ml.)

Solutions

Ferrous Sulphate. 20 g. of $FeSO_4.7H_2O$ are dissolved in distilled water and made up to 100 ml.

2.5 N-*Sodium Hydroxide.* 10 g. of NaOH are dissolved in water and made up to 100 ml.

Ehrlich's Dimethylaminobenzaldehyde Reagent. 0.7 g. of *p*-dimethylaminobenzaldehyde is dissolved in a mixture of 150 ml. of concentrated hydrochloric acid and 100 ml. of water.

Sodium Acetate. Saturated solution of sodium acetate.

[illegible]

made up to 100 ml.

THE STANDARD OF WATSON, SCHWARTZ, SBOROV AND BERTIE (55)

[illegible]

A QUALITATIVE TEST FOR UROBILINOGEN AND UROBILIN IN URINE (30)

Zinc Test

To 5 ml. of urine are added 2 drops of 0.1 N-iodine solution followed by 5 ml. of a 10% (w/v) suspension of zinc acetate in ethanol. The mixture is allowed to settle and in the clear supernatant a green fluorescence becomes apparent if urobilin or urobilinogen is present. If a spectroscope is available, the fluid may be examined for the broad absorption band (due to urobilin) at the green-blue junction.

Quantitative estimations of urobilinogen may be carried out using Ehrlich's reagent, dimethylaminobenzaldehyde, as described for the estimation of urobilinogen in faeces.

Significance of Estimations of Urobilinogen Excretion in Haemolytic Anaemia

haemolysis are probably better determined by red-cell survival studies rather than by the relatively inaccurate measurement of the amount of pigment excreted.

The amount of urobilin in the urine is not a reliable index of haemolysis, for excessive urobilinuria is an indication of liver dysfunction rather than a sign of increased red-cell destruction.

DEMONSTRATION OF HAEMOSIDERIN IN URINE

Method

in the acid-potassium ferrocyanide solution and allowed to stand at room temperature for 5 to 10 minutes. The suspension is then re-centrifuged. The deposit is transferred to a slide, covered with a cover-glass and examined under the microscope using the 4-mm. objective. Haemosiderin, if present, appears in the form of isolated or grouped blue-staining granules, usually from 1 to 3 μ. in size.

If a permanent preparation is required, the unstained urinary deposit is allowed to dry in the air. It is then stained by the same technique as is used to stain blood films for siderocytes (p. 75). The deposit is first fixed by dipping the slide in methanol for 10 to 20 minutes. It is stained in freshly prepared acid-potassium ferrocyanide solution for 10 minutes in the 56°C. water-bath. The slide is then washed in running water for 20 minutes, rinsed in distilled water and finally counterstained with 0.1% safranin or eosin.

Significance of Haemosiderinuria (13)

Haemosiderinuria is the consequence of haemoglobinaemia and the presence of haemoglobin in the glomerular filtrate. It is a valuable sign of

and the iron it contains then re-excreted.

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CHAPTER 9

L.E. CELLS AND LEUCOCYTE AGGLUTININS

THE L.E.-CELL PHENOMENON

Two hitherto unrecognized types of blood cell were described in bone-marrow preparations by Hargraves, Richmond and Morton (5) in 1948 as the "Tart" cell and the L.E. cell, respectively. It is now generally accepted that L.E. cells can be demonstrated, with possible rare exceptions (7a), only in bone-marrow and peripheral-blood preparations of patients suffering from disseminated lupus erythematosus (D.L.E.) (6). The presence of "Tart" cells, on the other hand, seems *not* to be correlated with any particular disease. They may in fact be found in health.

The L.E.-cell phenomenon depends on the presence in plasma of a γ globulin, the L.E. factor, which has the property of causing *in vitro* lysis of the nuclei of neutrophil polymorphonuclears and subsequent phagocytosis of the lysed nuclei by other neutrophils. The mechanism of the reaction is uncertain, one hypothesis is that the L.E. factor releases from combination with an inhibitor an enzyme, desoxyribonuclease, which is normally present in leucocytes and which has the property of causing depolymerization of the desoxyribonucleic acid of the nuclei of neutrophils (8). It is well known that L.E. cells cannot be found in films made from blood or bone-marrow immediately on withdrawal of the specimen; on the contrary they develop only on incubation *in vitro*, increasing in numbers up to 1½ to 2 hours. It seems in fact possible that only the nuclei of dead cells can be acted upon (12).

Coagulation has been thought to be important for their development, and Lee, Schwartz and Pariser (10) suggested that a substance derived from blood platelets, liberated during clotting, activates the L.E.-cell factor

Although the L.E.-cell phenomenon can be demonstrated using citrated, oxalated, heparinized or defibrinated blood (7), there is reason to believe that anticoagulants act as inhibitors and that L.E cells can be most frequently demonstrated, in the largest numbers, if clotted or defibrinated blood is used (4, 9)

L.E. cells can also be demonstrated if normal leucocytes are suspended in the serum of a patient with D L.E. (see below). Although it is convenient to use human leucocytes for this test, it has been claimed that the leucocytes from certain animals, such as the chicken, horse, guinea-pig and dog, are more sensitive to the L.E. factor than human cells (1).



FIG. 30. A TYPICAL L.E. CELL

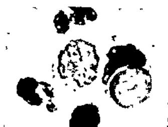


FIG 31 A "TART" CELL

A lymphocyte, with intact nuclear structure, has been engulfed by a monocyte, the nucleus of which has been compressed × 1,000

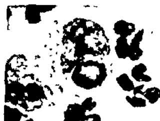


FIG 32 A "TART" CELL

A pyknotic (?) lymphocyte nucleus has been phagocytosed by a monocyte 1,000

The clinical and laboratory aspects of D.L.E have been recently well reviewed by Harvey and colleagues (6) They concluded that although L.E. cells are to be found in the majority of patients suffering from D L.E. (79 out of 96 personally studied patients), repeated examinations may have to be made before a positive result is obtained They stressed that the test may be negative throughout the illness in a small proportion of patients who appear clinically to be suffering from typical acute D L.E. The results of hundreds of tests for L.E. cells carried out on patients suffering from other diseases were uniformly negative.

Morphology of the L.E. Cell

In Romanowsky-stained preparations the L E cell appears as a neutrophil in the cytoplasm of which is a large spherical body (the L.E. body) which stains shades of pale purple The nucleus of the ingesting leucocyte is usually displaced to one side and may appear to be wrapped around the ingested material (Fig 30) The L E. body, although derived from nuclear material, usually shows no evidence of nuclear structure and appears as an opaque homogeneous mass The ingesting leucocyte is almost invariably a neutrophil polymorphonuclear, very rarely a monocyte or eosinophil

The "Tart"* cell is a monocyte—rarely a neutrophil—which has phagocytosed another cell or the nucleus of another cell (Fig 31). The phagocytosed material most often resembles a lymphocyte nucleus, in which case a definite nuclear pattern can be seen (Fig 31), however, a common alternative form is a pyknotic nucleus smaller than an L.E. body, and staining far more intensely (Fig 32) As already mentioned, the significance of "Tart" cells is not known

Demonstration of L E Cells

Two ways of demonstrating the L E -cell phenomenon will be described: (a) using defibrinated whole blood from the patient and (b) using the patient's serum and leucocytes obtained from a normal subject Method (b) is used when the patient's freshly withdrawn blood is not available. It can be successfully carried out on serum which has been stored for months at −20°C A recently described alternative technique (12), which is claimed to be simple and highly effective, is to incubate for 1 to 2 hours a drop of blood from the patient in contact with a dried concentrate of normal leucocytes

Method Using Defibrinated Blood

The blood is defibrinated in a 100-ml conical flask, as described on p. 2, or by any other equally successful method 10 to 20 ml of blood

* "Tart" apparently refers to the name of the patient in whom cells of this type were first seen.

are convenient volumes. The clot is removed and the sample is then incubated in the conical flask for 2 hours at 37°C. Two 75×8-mm. tubes are filled with the incubated blood and centrifuged at 3,000 r.p.m. (about 1,500 g) for 5 minutes. The buffy coats from each tube, with 1 to 2 mm. of underlying packed red cells and overlying serum, are then carefully transferred, using a Pasteur pipette into a Wintrobe haematocrit tube The latter tube is centrifuged without delay at 3,000 r.p.m. for 5 minutes. Films are made of the buffy coat and dried as quickly as possible by waving in the air They are fixed in methanol and stained with a Romanowsky dye

Method Using the Patient's Serum and Normal Leucocytes

Patient's Serum

This is obtained from blood allowed to clot undisturbed at room temperature or at 37°C. or from defibrinated blood. It should be stored frozen at −20°C. until used

Normal Leucocyte Suspension

5 ml. of freshly-drawn group-O blood are delivered into a container in which 1 mg. of heparin has been dried. After mixing, the blood is centrifuged at 3,000 r.p m for 5 minutes. The lower half of the column of packed red cells is then removed with a Pasteur pipette and discarded; the remaining red cells and the supernatant plasma are re-mixed, placed in a tube of about 8-mm. bore and allowed to sediment at 37°C. The removal of some of the red cells renders the blood anaemic and increases the rate of sedimentation.

The blood is allowed to sediment until 1 to 2 ml. of plasma are available This usually takes 30 to 60 minutes The supernatant plasma, containing leucocytes, platelets and a small number of red cells, is placed in a 75×10-mm tube and washed three times in saline. It is important to centrifuge the leucocyte suspension at a slow speed (500-1,000 r.p.m) and for no longer than 5 minutes. Before saline is added, it is essential to resuspend, by tapping the tube, the button of leucocytes in the small volume of fluid that remains after the supernatant fluid has been poured off. The leucocyte button is resuspended in the fluid remaining in the tube after the final washing and is then ready for use

Technique of Test

1 ml of the serum to be tested is added to the tube containing the small volume of the resuspended washed leucocytes. The mixture is incubated at 37°C. for 2 hours. The tube is then centrifuged and the supernatant serum removed by Pasteur pipette. The deposit of leucocytes is broken up by gently sucking it up and down the pipette. Films of the leucocyte suspension are then made, and are dried, fixed and stained with a Romanowsky dye in the usual way.

Examination of Films

Each slide should be examined for at least 10 minutes before a negative report is given. With practice it is possible to recognize L.E. cells using a 16-mm. objective. Free extracellular masses of altered nuclear material or masses surrounded but not ingested by neutrophils (rosette formation) may be seen, but these should not be considered diagnostic unless the characteristic L.E. cells are also seen. The majority of L.E. cells are usually found at the edges of the films.

The number of L.E. cells found varies within wide limits. Occasionally large numbers are present; more often scattered cells are found only after prolonged search. If sufficiently numerous, they may be reported as the number present per 1,000 neutrophils. Tart cells can usually be clearly differentiated from L.E. cells, but it must be admitted that they are occasionally a source of difficulty.

Both the techniques described above are sensitive. The technique using defibrinated whole blood is the simplest and should be used first. If results with this test are repeatedly negative, the method employing normal leucocytes and the patient's serum should be tried.

There does not appear to be any advantage in using bone-marrow for the purpose of demonstrating L.E. cells, since peripheral blood techniques are sensitive and can be repeated frequently without discomfort to the patient.

DEMONSTRATION OF LEUCO-AGGLUTININS

Within recent years evidence has been accumulating that some cases of spontaneous or drug-induced granulocytopenia are brought about, at least in part, by leucocidal or leuco-agglutinating antibodies (2, 3, 11). Some of the characters of the leuco-agglutinating antibodies and details of technique are described by Dausset (2) and Dausset, Nenna and Brecy (3). It should be added that in addition to the agranulocytoses, leuco-agglutinins have been demonstrated in the serum of patients who have had many transfusions, particularly in "pancytopenia", paroxysmal nocturnal haemoglobinuria, acquired haemolytic anaemia and various types of leukaemia (2).

Basically, Dausset's test (2) consists of adding a suspension of normal leucocytes freshly obtained from defibrinated blood to the patient's heat-inactivated serum (or preferably to dilutions of the patient's inactivated serum so as to guard against the possibility of a prozone). The heating at 56 C for 30 minutes is necessary as fresh serum apparently contains an inhibitor. The mixture is incubated at 37°C. for 1 hour and the suspension of leucocytes viewed microscopically after adding a small volume of 1% acetic acid to lyse any red cells present. For additional technical details and illustrations of positive tests the paper by Dausset, Nenna and Brecy (3) should be consulted.

Tests for leuco-agglutinins seem likely to be useful in haematological

research, if not in routine practice. However, it is doubtful whether the best method of demonstrating the antibodies has yet been discovered. The techniques suggested up till now, although simple in theory, need to be very carefully carried out, and the results must always be compared in parallel with those obtained with control normal sera. *The significance of positive leuco-agglutinin tests* in patients with leucopenia remains to be determined. The agglutinins may act as auto-leuco-agglutinins but this has not been proved. The possibility that they represent intra-group iso-leucocyte antibodies has not been excluded, and it is perhaps significant that most patients in whom leuco-agglutinins have been observed had previously been transfused.

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CHAPTER 10

INVESTIGATION OF THE HAEMORRHAGIC DISORDERS

IN this chapter will be described the laboratory techniques which may be used in the investigation and differentiation of the haemorrhagic disorders, with some indications as to the value of the tests and the order in which, in the author's opinion, they should be undertaken. The investigation of the blood-coagulation mechanism will be described first, and this will be followed by a description of methods for measuring the bleeding time and studying the blood platelets No attempt will be made to describe the theory of blood coagulation in detail. The nomenclature used conforms to the usual British practice (6, 7)

INVESTIGATION OF THE BLOOD-COAGULATION MECHANISM

Collection of Blood

Blood should be taken from a vein into a syringe, preferably coated with silicone,* using a relatively short needle of 19 or 20 S W.G The venepuncture must be a "clean" one and the blood should be withdrawn slowly without allowing frothing to occur It is convenient to distribute the blood into tubes as follows, after detaching the needle from the syringe

(1) Four 1-ml. samples are delivered into small (75×8-mm) tubes, previously warmed at 37°C, for the measurement of the whole-blood coagulation time, (2) 3 to 4 ml are placed in a 75×12-mm tube containing several glass beads to provide a source of serum—the tube should be inverted several times before the blood has firmly clotted, (3) the remainder is citrated by adding nine parts of blood to one part of 3·8% trisodium citrate in a siliconed centrifuge tube

The serum from the tube containing the glass beads is used in the thromboplastin-generation test, and the citrated plasma is required for the measurement of "prothrombin" time, as a reagent in the thromboplastin-generation test, and as a source of platelets Pooled serum from the coagulation tubes can be used for the prothrombin-consumption test A small volume of blood should also be delivered on to a waxed watch glass so that the platelets can be counted and blood films prepared (see p 37) Alternatively, a measured volume of blood can be delivered into a bottle containing Sequestrene (see p. 3)

* See p 222 for the preparation of siliconed apparatus

THE WHOLE-BLOOD COAGULATION TIME

The estimation *in vitro* of the coagulation time of blood is an artificial procedure, and the results depend very much upon the particular technique used. Amongst the variables which need to be controlled are the method of obtaining the blood, the size and nature of the vessel into which the blood is placed, the temperature, and how the end-point of coagulation is determined

Only by the use of venous blood is it possible to measure the coagulation time at all reliably. A method based on that of Lee and White (22) will be described in detail. However, the method of Dale and Laidlaw (9) will also be described because it may be necessary to use peripheral blood, even if it is less satisfactory, should a clean venepuncture prove to be impracticable in small children.

Method Based on that of Lee and White (22)

Venous blood is withdrawn into a dry syringe and, after detaching the needle, 1-ml. volumes are delivered into four clean unsiliconed tubes of 8-mm. internal bore previously warmed to 37°C. (It is immaterial for this test whether or not the syringe is siliconed.) A stop-watch is started as soon as the blood enters the syringe. The tubes in rotation are then gently tilted every minute until one can be tilted through an angle greater than 90° without spilling the blood. The remaining tubes are then examined at half-minute intervals (or, if the coagulation time is abnormally prolonged, at longer intervals). The coagulation time is taken as the average of the clotting times in all four tubes or, alternatively, if the coagulation time is greatly prolonged, as the time necessary for the blood to clot in at least two of the four tubes.

Chemically clean tubes are essential and the venepuncture must be carried out and the blood handled as described on p. 151.

It is important to control the temperature as the speed of clotting increases with the temperature—it is about twice as fast at 37°C. as at average room temperature (20°C.) For this reason it is best if the patient can be brought to the laboratory. If this is impracticable, a glass jar with a pierced metal lid, containing water at 37°C., can be taken to the bedside The tubes, which should be rimmed, may be dropped through the holes in the lid and supported by their rims. A separate hole for a thermometer is useful.

Measurements at room temperature are less satisfactory, coagulation is needlessly prolonged and the temperature itself is too variable The size of the container must be standardized, for blood clots faster in narrow tubes because of the increased surface of blood in contact with the wall of the container. The tubes must be perfectly clean and there seems no point in using, in routine investigations, tubes coated with silicone so as to retard coagulation

The exact measurement of the end-point is a matter of some difficulty

Blood clots first at the periphery in contact with the glass container and at the surface exposed to air. It clots last in its centre. It is essential, therefore, in reading the end-point to tilt the tubes gently and always in the same way if standard results are to be achieved

When coagulation is markedly prolonged, as it may be in haemophilia, the reading of the end-point is particularly difficult. Quick and co-workers (34), for instance, cited 10 minutes as the time of commencing coagulation of a particular sample of haemophilic blood, although coagulation was not complete enough to form a solid clot until 2 hours had elapsed

Normal Range for the Whole-Blood Coagulation Time Estimated by Lee and White's Method (22, 27)

5 to 11 minutes (usually 6 to 9 minutes) at 37 C

Method of Dale and Laidlaw (9)

A warmed ear or finger is deeply punctured, so as to obtain a free [illegible] tube is then grasped at each end by forceps the blades of which are covered by a short length of rubber tubing and is dipped into water warmed to 37°C The tube is slowly tilted up and down and the move- [illegible] the water-bath or beaker in which the tube is immersed

A rapid flow of blood from the puncture is absolutely essential, and if squeezing or manipulation is required to obtain the blood the test should be abandoned The method is undoubtedly insensitive and should not be used if venous blood can be obtained It may give normal results at a time when the Lee and White method shows a definite prolongation in clotting time

The *normal coagulation time* is up to 3 minutes

Significance of the Measurement of the Whole-Blood Coagulation Time

The estimation *in vitro* of the whole-blood coagulation time is only of limited value even if a careful technique is followed. As only small amounts of thrombin are required to clot fibrinogen, the test is inevitably insensitive For instance, the coagulation mechanism may be grossly impaired by drugs such as Tromexan or dicoumarol, or in haemophilia, without this necessarily raising the coagulation time above

the normal range. Similarly, fibrinogen must be markedly deficient before the blood fails to clot because of fibrinogenopenia.

The test is "non-specific" in the sense that several quite distinct abnormalities, if sufficiently severe, lead to a prolongation of the whole-blood coagulation time. However, it is relatively sensitive to defects in the mechanisms which act at an early stage of coagulation For this reason prolongation of the whole-blood coagulation time (other [illegible] or (more rarely) the presence of circulating anticoagulants.

RECALCIFICATION TIME ("CALCIUM TIME")

Principle. If calcium is added to citrated plasma, a fibrin clot develops after a variable interval of time. The time which elapses between the addition of the calcium and the appearance of the clot is the recalcification time.

Method

Citrated blood is obtained, as described on p 151, and lightly centrifuged in a silicone coated tube at a speed of 1,000 r.p m (about 200 *g*) for not longer than 2 minutes to obtain plasma rich in platelets. 0·1 ml. of the plasma and 0·1 ml. of saline are delivered into a chemically-clean 75×8-mm. tube previously warmed to 37°C. in a water-bath, 0·1 ml of 0·025 M-calcium chloride is then added to the mixture and a stop-watch is started The time taken for the plasma to clot is recorded.

Normal Range (6): 90 to 250 seconds.

Significance of the Recalcification Time

Like the whole-blood coagulation time this simple test is inherently inaccurate and difficult to standardize. The results are affected by the numbers of platelets present to a greater extent than is the whole-blood coagulation time It is imperative, therefore, that the plasma used for the test be obtained from lightly centrifuged blood It is also important to use siliconed glassware up to the time of the actual test—which is carried out in a plain glass tube—because contact with a water-wettable surface shortens the recalcification time considerably. The test should be carried out as soon as possible after the blood is collected because of the possibility of activation of the platelets on storage with consequent shortening of the recalcification time (17a)

The recalcification time is prolonged in all the conditions in which the whole-blood coagulation time is prolonged, and also in the presence of thrombocytopenia. The test, being essentially non-specific, has no particular clinical importance However, it does form the basis of the

thrombin-generation test (see below) which, if not important from the diagnostic point of view, demonstrates effectively the progress of thrombin formation. The method can also be used to demonstrate deficiency of a particular clotting factor by omitting the volume of saline and adding instead normal or pathological plasma or a plasma fraction to the patient's plasma before recalcification. Haemophilia can be differentiated from Christmas disease in this way. However, this can only be done if known haemophilic or Christmas-disease plasma is available (cf., the thromboplastin-generation test, p 168)*

THE THROMBIN-GENERATION TEST (24, 31)

Principle. The appearance of a clot when plasma is recalcified is not the end-point of the reactions taking place in the plasma. Normally, thrombin continues to be generated in increasing amounts for some time after clotting has occurred. The rate of generation then diminishes and the thrombin concentration drops because of the action of antithrombin. By transferring samples of the recalcified plasma at regular intervals into tubes containing a solution of fibrinogen, the concentration of thrombin at any particular moment can be inferred from the speed of clotting of the fibrinogen. The concentration of thrombin is inversely proportional to the time which it takes to clot fibrinogen. By reference to a correlation graph prepared by adding known dilutions of a solution of thrombin to tubes containing the fibrinogen solution, the thrombin concentration in the recalcified plasma, at the time of taking any particular subsample, can be expressed in "units" of thrombin. In this way a thrombin-generation curve can be prepared which illustrates the generation of thrombin and its subsequent decay in plasma which has been recalcified (Fig 33)

Method

0 2 ml of citrated plasma, obtained as for the recalcification time, and 0 2 ml of saline are placed in a 75 × 12-mm plain tube kept at 37° C in a water-bath. 0 2-ml. volumes of a fibrinogen solution (see below) are delivered into each of ten 75 × 8-mm plain tubes, also kept at 37° C in a water-bath. After the temperature of the diluted plasma has reached that of the water-bath, 0 2 ml of previously warmed 0 025M-calcium chloride solution is rapidly added to it. The contents of the tube are rapidly mixed by inversion and a stop-watch started. At 1-minute intervals, 0 05 ml of the reacting mixture is transferred using a calibrated Pasteur pipette to successive fibrinogen tubes. The clotting times of the fibrinogen are recorded. It is convenient to have an assistant who can record the clotting times, but this is not essential.

At a time which corresponds to the recalcification time of the plasma, the reacting mixture itself clots. The clot should be pressed against the wall of the tube with a wooden swab-stick and removed on the stick as rapidly as possible. Difficulty is sometimes experienced when the clotting time is delayed, as for instance with haemophilic plasma, for the fibrin may form in successive waves over a considerable period, so that repeated extractions with a wooden stick are necessary. Finally, the fibrinogen clotting times are converted from the correlation graph into units of thrombin (see p 157)

* A presumptive diagnosis of haemophilia (or Factor-V deficiency) can be made by showing that $Al(OH)_3$-treated normal plasma, but not normal serum, will shorten the clotting time of the patient's plasma. The converse is true in Christmas disease (or Factor-VII deficiency)

The Quick One-Stage Test

0·1 ml of plasma is delivered to the bottom of a 75×8-mm. tube placed in a water-bath at 37°C. and 0·1 ml. of the brain-extract suspension added to it. After a delay of 1 minute, 0·1 ml. of warmed 0·025M-calcium chloride is added and the contents of the tube are quickly mixed. A stop-watch is started and the tube is carefully watched with the lower end just below the surface of the water. A fibrin clot appearing quite suddenly within a second or two marks the end-point. The test should be repeated once or twice, the tube being lifted clear of the water a few seconds before the end-point is expected in order to facilitate inspection. The average time for the appearance of the fibrin is recorded as the prothrombin time. A normal control plasma should be tested in the same way at the same time.

In the above technique, 3·8% sodium citrate or dry ammonium and potassium oxalate mixture (2) is substituted for the sodium oxalate solution recommended by Quick (33) and human brain for rabbit brain. *One advantage of using human brain is that large batches of* thromboplastin can be made at the same time, if suitably stored, their potency will keep for months (see later).

Reagents

Patient's and Control Plasma The blood must be obtained by a clean venepuncture. There is no need to use a siliconed syringe or glassware unless other tests, such as the thromboplastin-generation test, are to be carried out on the same blood The blood may be citrated (p. 151) or, alternatively, 5 ml. of venous blood may be added to 10 mg. of potassium and ammonium oxalate mixture and gently mixed with the anticoagulant by several inversions of the tube Part of the sample can then be used for haemoglobin or P C V estimation, etc *The citrated or oxalated blood should be centrifuged without delay at 1,500* r.p.m. for 5 to 10 minutes The supernatant plasma is then removed; if not used at once it should be placed in the refrigerator at 4°C. where it may remain for several hours With oxalated plasma the test should be carried out with as little delay as possible, however, with citrated *plasma there* seems to be less need to hurry, because of the relative stability of Factor V in citrated plasma In any case, the *estimations* must be carried out on the day the plasma is collected

Human Brain Extract. An acetone extract of brain is generally used (for preparation, see p 160) 0 3 g of the dry *brain extract is* suspended in 5 ml. of saline and heated to 37°C for 15 to 30 minutes, with occasional shaking, so as to get the active material into solution. The coarse particles are allowed to sediment and the opalescent super-*natant is used for the test.* The solution may be kept for a day or two at 4°C or longer if frozen at −20°C

Normal Range of Prothrombin Time: 10–14 seconds.

SIGNIFICANCE OF RESULTS OBTAINED BY THE QUICK ONE-STAGE TEST

The speed of clotting in the prothrombin-time test depends on the concentrations of Factor V and Factor VII in the plasma as well as on that of prothrombin. Indeed, the increased prothrombin time caused by dicoumarol or Tromexan is due more to deficiency of Factor VII than to reduction in prothrombin concentration (10). This is probably also true of the prolonged prothrombin times sometimes found in severe liver disease. The one-stage prothrombin-time test is therefore [illegible]

brain extract is reliable (28). From the laboratory point of view the one-stage test occupies an important place in the routine investigation of coagulation disorders, for if it gives a normal result major deficiencies of Factor V and Factor VII can be excluded.

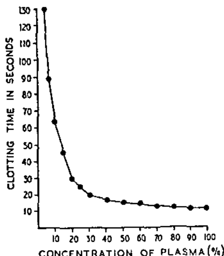


FIG 35 PROTHROMBIN-TIME DILUTION CURVE

The graph illustrates the effect on its prothrombin time of the progressive dilution of a normal plasma with saline

Recording Results of the Prothrombin-Time Test

The results of the test can be recorded in one of three ways: (I) as a time in seconds, (II) as a "prothrombin" index (P.I.), e g, $\frac{\text{prothrombin time of control plasma}}{\text{prothrombin time of patient's plasma}} \times 100$, (III) as a percentage based on the results of previously constructed dilution curves made diluting normal plasma with saline using the same batch of brain ex

Method (III) works well in practice and seems to be most generally used. Method (II) is also quite reliable (28), but care must be taken not to confuse a P.I. expressed as a percentage with percentage of "prothrombin activity" based on Method (III). The effect on the prothrombin time of progressive dilution of a normal plasma with saline is illustrated in Fig. 35. If the clotting times are plotted on ordinary graph paper against the reciprocals of the plasma dilutions the observations approximate to a straight line. If a series of different normal plasmas are diluted, the prothrombin times estimated, and the results plotted as above, then an approximate range of percentage prothrombin activity can be read off for any particular observed clotting time; e.g., clotting time 18 sec., prothrombin activity = 21 to 30%. The standardization requires to be repeated for each new batch of thromboplastin.

Preparation of Human-Brain Extract

A human brain freshly obtained from the postmortem room is stripped completely of its covering membranes and blood vessels. It is then cut into small pieces and macerated in acetone in a mortar. After the acetone has been changed several times a non-adhesive granular material remains. This is crude "acetone-brain" extract.

The granular material is dried in an evacuated desiccator and when dry 0.3-g. amounts are placed in a number of 75×12-mm tubes provided with cotton-wool plugs. These are stored in an evacuated desiccator at 4°C until used. Under these circumstances they retain their potency for months. The desiccator must be re-evacuated each time it is opened to remove a tube.

Use of the Quick One-Stage Prothrombin-Time Test in the Detection of Factor-V or Factor-VII Deficiency

Qualitative Tests

Deficiency of Factor V can be demonstrated in two ways

[illegible] plasma of one volume [illegible]

[illegible] a substantial shortening in the one-stage prothrombin time [illegible]

VII, the addition tially shorten the

xan ("Tromexan-plasma") as a substrate and comparing the effect of adding to it one-tenth volumes of normal plasma and the patient's plasma, respectively. If the patient's plasma lacks Factor VII, the one-stage prothrombin time of the Tromexan plasma will be shortened to a substantially less extent, if at all, by the patient's plasma compared with the normal plasma.

Quantitative Test for Factor-V Deficiency

One volume of normal plasma is added to nine volumes of Factor-V

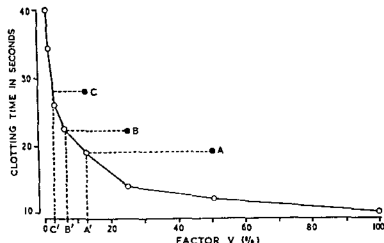


FIG 36 GRAPH ILLUSTRATING THE ESTIMATION OF FACTOR V

mean = 28%

Quantitative Test for Factor-VII Deficiency

One volume of normal plasma is added to nine volumes of Tromexan plasma, and the one-stage prothrombin time estimated. The test plasma is then substituted for the normal plasma. If the clotting time with the test plasma is longer than with the normal plasma, dilutions of the normal plasma are made in saline and the one-stage prothrombin times of the Tromexan plasma, to which the dilutions of normal plasma have been added, estimated. A curve can then be constructed from which the approximate concentration of Factor VII in the test plasma can be read off.

Principle of the Method of Owren (29a and b)

This method differs from that of Quick in that Factor V and fibrinogen are added to the test plasma which is used diluted. The method is a sensitive one and, although more complicated than that of Quick, it is particularly useful when tests cannot be carried out within a short while of the blood being collected.

Method

0·2 ml. of prothrombin-free ox plasma is mixed in a 10-mm. tube with 0·2 ml. of diluted test (or control) plasma and 0·2 ml. of thromboplastin solution. After 5 minutes at 37°C., 0·2 ml. of the calcium chloride solution is added, and the clotting time of the mixture measured by means of a stop-watch.

Recording Results

The clotting time of the test plasma can be converted into "per cent prothrombin and proconvertin (Factor VII) activity" from a correlation graph. Normal plasma is diluted from 1 in 10 to 1 in 100 in veronal buffer containing 30 mg. per 100 ml. of potassium oxalate and the clot- [illegible] as described in the previous paragraph [illegible] d [illegible] is technique for following the progress of a patient on anticoagulant therapy it is convenient to construct a calibration graph using as the normal plasma the patient's plasma withdrawn before the start of treatment.

Reagents

Test and Control Plasmas

4·5 ml. of venous blood are rendered incoagulable by adding the blood to 0·5 ml. of 2% potassium oxalate (29a). After centrifugation, one volume of plasma is diluted with nine volumes of the veronal buffer.

Prothrombin-Free Ox Plasma

This reagent supplies Factor V and fibrinogen. Nine volumes of fresh ox blood are rendered incoagulable with one volume of 2·5% potassium oxalate. Prothrombin and Factor VII are then removed by passing the ox plasma once through a clarifying filter-pad containing 20% asbestos and then

through a filter containing 50% asbestos (29b) The pH of the plasma is adjusted to 7 3 by the addition of 0 5 N-HCl using an external indicator. The plasma is then stored frozen at −20°C. in small, e g , 2- or 4-ml volumes *It retains its potency for several months*

Alternatively, oxalated ox plasma adsorbed with barium sulphate* may be used in the test as a source of Factor V and fibrinogen instead of filtered ox plasma

Thromboplastin (saline extract of human brain)

Human brain is freed from its membranes and blood vessels It is washed

each test

Veronal Buffer, pH 7 35, see p 221

Calcium Chloride

The optimal concentration should be determined for each batch of ox plasma 0 03M-$CaCl_2$ is usually satisfactory

ESTIMATION OF PROTHROMBIN BY A TWO-STAGE METHOD (3, 6)

Principle Brain extract is added to citrated plasma which is then recal

against the times of sub-sampling, they form a curve showing as a rule a sharp rise in thrombin content, then a fall The area under the curves given by a normal and the test plasma are compared

Thrombin is normally very rapidly generated, but it is almost as rapidly destroyed as the result of the action of plasma antithrombin It has to be assumed for the purposes of the test that the antithrombin activity of the normal and test plasma are similar When Factors V and VII are deficient, the generation of thrombin is slow, but it persists longer so that the area under the curve obtained experimentally is not greatly reduced In true prothrombin deficiency, on the other hand, thrombin is generated at the normal rate, but it never achieves a high concentration and disappears unusually quickly (3) (Fig 37)

Method

0 4 ml of citrated fresh normal plasma is placed in a 75×12-mm tube in a water-bath at 37°C and 0 4 ml of brain extract is added to it A series of *ten 75×12-mm tubes containing 0 4 ml of human or bovine fibrinogen solution at a concentration of 100 to 200 mg per 100 ml are also placed in*

* 50 mg of barium sulphate are added per 1 ml of plasma The adsorbed plasma is centrifuged after 10 to 15 minutes at 37°C X-ray barium sulphate is suitable but it should be first washed twice in 0 005M-trisodium citrate to remove the finer particles (Biggs, *personal communication*)

the water-bath. After allowing several minutes for the temperature of the mixture to reach that of the water-bath, 0 4 ml of 0 025M-$CaCl_2$ is added. A stop-watch is started at the moment of the addition of the calcium, and 30 and 60 seconds later, and subsequently at 1-minute intervals, sub-samples of the mixture are transferred to the tubes containing the fibrinogen. The clotting times of the fibrinogen samples are recorded, and the experiment continued [illegible]

At a [illegible]
plasma [illegible]
as quick [illegible]
and pressed against the side of the tube so as to squeeze the clot. An inexperienced worker will find the help of an assistant, who can record the fibrinogen clotting times, an advantage.

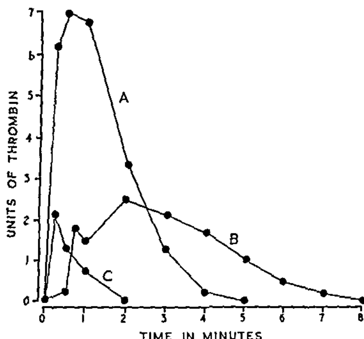


FIG 37 ESTIMATION OF PROTHROMBIN BY A TWO-STAGE METHOD

Curve A Result with normal plasma *Curve B* Result with plasma from a patient receiving Tromexan *Curve C* Result with the plasma of a patient suffering from true prothrombin deficiency (Redrawn from Biggs and Douglas (1953))

The fibrinogen clotting times, converted to units of thrombin (p 157), are plotted against the times of sub-sampling on arithmetical graph paper

The whole experiment is repeated using the test plasma The areas enclosed by the two thrombin-generation graphs are compared, measuring the areas with a planimeter or by counting the small squares on the graph paper The prothrombin percentage is given by

$$\frac{\text{area under curve given by test plasma}}{\text{area under curve given by normal plasma}} \times 100.$$

THE PROTHROMBIN CONSUMPTION TEST

Principle. During normal coagulation, thrombin production (and prothrombin utilization) continues after the blood or plasma has clotted. If the serum is tested 1 hour after coagulation, it will be found that practically all the prothrombin has been " consumed ". In most disorders of coagulation, and in thrombocytopenia, the formation of " intrinsic " thromboplastin is impaired (24), and this leads to incomplete utilization of prothrombin, even though the blood or plasma may clot in the normal time

The prothrombin consumption test has been widely used clinically (26, 35, 37) as a measure of prothrombin consumed; it is a fairly delicate —but quite non-specific—measure of clotting efficiency. In the test described below the prothrombin in plasma, and in serum, is measured by a two-stage procedure.

Reagents

I. Citrated plasma (see p. 151)

II. Serum. This is separated from clotted blood 1 hour after coagulation has taken place It should be obtained from the blood used for the whole-blood coagulation time, not from blood clotted in the presence of glass beads 1 hour after coagulation is selected as a standard time to carry out the test If the actual estimation cannot be carried out 1 hour after clotting, the serum should be citrated with a one-tenth volume of 3 8% (w/v) trisodium citrate so as to inhibit further conversion of prothrombin

III Human brain extract (p 160)

IV. 0 025M-calcium chloride

V. Human or bovine fibrinogen, at a concentration of 150 to 200 mg. per 100 ml

Method

Sufficient calcium chloride solution and fibrinogen are placed in the water-bath at 37°C before the actual test is started 0 1 ml of plasma is then delivered into a 75 × 12-mm tube and 0 1 ml. of brain extract added to it After waiting a minute or so for the plasma and thromboplastin to warm, 0 1 ml of 0 025M-calcium chloride is added to the mixture and a stop-watch started. After exactly 60 seconds, 0 2 ml. [illegible]

clot The clot has to be removed on a wooden swab-stick before the fibrinogen is added (It is convenient to put the swab-stick in the [illegible] at the start of the experiment)

The test is repeated in exactly the same way using serum instead of plasma. Naturally, in this instance, there will be no clot to remove before the fibrinogen is added, except perhaps in severe haemophiliacs.

Reporting Results

The relationship between the plasma and serum clotting times is expressed as an index; i.e., prothrombin consumption index (P.C.I.)

$$= \frac{\text{plasma clotting time}}{\text{serum clotting time}} \times 100.$$

This method of reporting suffers from the fact that *low* percentages are normal, whilst a value of 100% is grossly abnormal. For this reason some workers are content to record simply the clotting time using serum as the "serum prothrombin time" or "serum prothrombin activity" (37).

Normal Range of Prothrombin Consumption Index (P.C.I.): 0 to 40% (serum prothrombin time >30 seconds).

THE THROMBOPLASTIN-GENERATION TEST (4)

Principle Four components essential for the formation of thromboplastin in plasma are prepared separately and their ability to form thromboplastin when mixed and incubated together tested by adding sub-samples of the mixture to normal prothrombin-containing plasma. The four components are (*a*) Factor V and antihaemophilic globulin (AHG), as contained in $Al(OH)_3$-treated plasma; (*b*) Factor VII and Christmas factor (as contained in serum); (*c*) washed platelets, and (*d*) calcium. By varying the source of the separate components *a*, *b* and *c*, it is possible to test the clotting efficiency of each; e.g., a patient's plasma or serum can be tested for the presence of AHG or Christmas factor or a patient's platelets tested for their ability to take part in the formation of thromboplastin

Reagents

Plasma Both normal plasma and the patient's plasma are treated in the following way The blood should be collected with a siliconed syringe, and added to a one-tenth volume of 3 8% trisodium citrate in a siliconed tube (p 151). The plasma serves as a source of platelets as well as of AHG and Factor V, and provides the substrate on which the thromboplastin activity is tested

Part of the plasma is centrifuged at 2,000 to 3,000 r.p.m for 15 minutes, and to 1 ml of this plasma is added 0 1 ml of aluminium-hydroxide gel (p 221) After mixing and allowing the mixture to stand at 37°C for 5 minutes, the plasma is again centrifuged, and the supernatant preserved It should contain Factor V and AHG, but little or no

prothrombin or Factor VII—if brain extract and calcium are added to it, the clotting time should lie between 60 and 240 seconds. The $Al(OH)_3$-treated plasma is diluted 1 in 5 in veronal buffer at pH 7·35 (p. 221) before use

Serum This should be obtained as described on p 151, making sure that the blood is brought effectively into contact with the glass surface of the tube during the initial stages of clotting After being allowed to stand for 2 to 4 hours at 37°C., the separated serum is centrifuged to free it of suspended cells It is diluted 1 in 10 in veronal buffer before use. Both normal serum and patient's serum are required

Platelets. The patient's plasma can be used as a source of platelets if they are known to be normal If not, a suspension of platelets from a normal subject must be prepared When dealing with a patient suffering from haemophilia there is a theoretical advantage in using the patient's platelets, for it is difficult to wash platelets completely free from plasma, which normally contains AHG However, in practice, normal platelets are satisfactory

10 ml. of citrated plasma (of normal platelet count) collected into a siliconed tube, are required to supply sufficient platelets The plasma is first centrifuged at a relatively slow speed (e.g, 1,500 r.p m) for 10 minutes and the platelet-rich supernatant plasma then transferred to a clean siliconed tube and centrifuged at a higher speed, e g, 3,000 r.p.m. for 15 minutes The deposited platelets are washed twice with saline, breaking up the deposit each time with a wooden swab-stick. The platelets are finally resuspended in a volume of saline one-third of the volume of the plasma from which they were derived The platelet suspension may be kept frozen at −20°C for several days, or perhaps even weeks, before being used, without apparently losing its potency The supernatant plasma is preserved and used as substrate (see later).

Calcium 0 025M-calcium chloride

SUBSTITUTES FOR PLATELETS

The possibility of using a brain extract as a more permanent substitute for platelets has been investigated by Bell and Alton (2a) Preliminary trials with the extract are encouraging It is made as follows

1 g of acetone-dried human brain (see p 160) is subjected to a further extraction with 20 ml of acetone and the mixture centrifuged after being allowed to stand at room temperature for 2 hours The supernatant is discarded and the sedimented brain tissue dried in an evacuated desiccator

trial

More recently, Newlands and Wild (28a) have used soybean phospho in place of platelets and have found this to be satisfactory in the d haemophilia and Christmas disease.

Method

0·1 ml. of normal citrated plasma is delivered into six 75×12-mm. tubes placed in a water-bath at 37°C. As mentioned above, if the platelet suspension has been made from normal plasma, the residual plasma can be used as substrate.

In another tube are placed 0·2-ml. volumes of: (*a*) the $Al(OH)_3$-treated plasma (1 in 5); (*b*) serum (1 in 10); and (*c*) platelet suspension. After allowing the mixture to warm to the temperature of the water-bath, 0·2 ml. of 0·025M-calcium chloride is added and a stop-watch started. At 1-minute intervals, 0·1 ml. of the incubating mixture is transferred to successive tubes containing plasma, and at the same time, using the other hand, 0·1 ml. of 0·025M-calcium chloride solution is added to successive tubes. The clotting times of the plasma samples are recorded.

Recording Results

It is usually sufficient to record the substrate clotting times in seconds corresponding with the period of incubation of the mixture forming thromboplastin (Table XIII). Alternatively, the clotting times of the plasma samples can be converted into "per cent thromboplastin activity" as follows:

Using normal reagents, after 4 to 5 minutes' incubation, sufficient thromboplastin is usually generated in the incubation mixture to cause the plasma substrate to clot in 8 to 10 seconds. If the incubation mixture is then placed in ice-water at this point to slow down the further progress of the reaction and dilutions made of it—ranging from 5% to 90%—the plasma clotting times corresponding to the thromboplastin dilutions can be determined. A curve is obtained which can be used in subsequent work to convert clotting times to per cent thromboplastin activity.

PRACTICAL USE OF THE THROMBOPLASTIN-GENERATION TEST (32)

The great virtue of the thromboplastin-generation test is that it allows the efficiency of different components of the clotting system to be tested separately. In haemophilia, for instance, it will be found that thromboplastin generation is more or less subnormal if plasma derived from the patient is used (the other reagents being derived from a normal subject), whereas it is normal if his serum is used. In Christmas disease, the exact opposite will be found. Indeed, by this test it is possible to demonstrate minor degrees of haemophilia (AHG deficiency) which do not seem to be demonstrable with certainty by any other laboratory test yet available. The data obtained from tests carried out on three patients suffering from haemophilia, Christmas disease and from a circulating anticoagulant, respectively, are illustrated in Table XIII.

As in the recalcification-time test (p. 154) the thromboplastin-generation test can be used to study the effect of adding normal or pathological $Al(OH)_3$-treated plasma, or serum, in various concentrations to the patient's $Al(OH)_3$-treated plasma, or serum.

TABLE XIII

REPRESENTATIVE RESULTS OF THROMBOPLASTIN-GENERATION TESTS IN HAEMOPHILIA, CHRISTMAS DISEASE AND IN THE PRESENCE OF A CIRCULATING ANTICOAGULANT

Mixture of reagents	Incubation time at 37°C (minutes)					
	1	2	3	4	5	6
	Haemophilia					
Normal plasma, Patient's serum, Normal platelets	12	9½	8	7½	7	7
Patient's plasma, Normal serum, Normal platelets	40	38	28	27	28	28
Normal plasma, Normal serum, Normal platelets	31	10	8	8	9	9½
	Christmas Disease					
Normal plasma, Patient's serum, Normal platelets	32	25	23	20	23	22
Patient's plasma, Normal serum, Normal platelets	31	10	8	8	9	9
Normal plasma, Normal serum, Normal platelets	31	15	9½	8½	9	9
	Circulating Anticoagulant					
Normal plasma, Patient's serum, Normal platelets	37	16	12	12	12	12½
Patient's plasma, Normal serum, Normal platelets	70	60	60	60	45	45
Normal plasma, Normal serum, Normal platelets	60	19	10	10	10½	—
Equal parts of normal plasma and patient's plasma, Normal serum, Normal platelets	60	40	26	23	22	22

The recorded figures represent the clotting times in seconds of the substrate to which the samples of the incubation mixture have been added

Assay of Antihaemophilic Globulin (AHG)

tes
as
in
method is less elaborate, but a supply of plasma from a known severely-affected haemophiliac is required. However, not more than 1 ml. of this plasma is used in each test and as it can be kept frozen at −20°C. for several weeks at least without spoiling, the test is quite a practical one. The method is described below

Pitney's Method (30)

a standard normal plasma

Reagents

I. Haemophilic Plasma. Blood is withdrawn from a severely affected haemophiliac and added to a one-tenth volume of 3 8% sodium citrate. It is centrifuged without delay at 3,000 r p m. for 15 minutes The supernatant plasma is then stored frozen at −20°C in 1-ml volumes Before the actual test a 1-ml. volume is adsorbed with 0 1 ml. of aluminium-hydroxide gel (see p. 221) The plasma supplies Factor V and should contain only

4°C. in the meanwhile

III. Platelet Suspension This is obtained as for the thromboplastin-generation test (p 167)

IV. Normal Plasma This must be obtained freshly for each assay The
is treated with
to give concen-
tion is referred to
ntains 1% AHG.
ined, it is treated
), 1 in 20, 1 in 40

Method

plasma *(I)*, 0 1 ml. of
) 1 ml. of
) 1 ml of
to 37°C.
started
1 ml of
thrombo-
to clot,

using each dilution of (*IV*), is then plotted on graph paper against the corresponding AHG content (i e , the dilution of the normal plasma). This gives a calibration curve against which the activity of the patient's (test) plasma can be compared.

[illegible]

DETECTION OF CIRCULATING ANTICOAGULANTS

Circulating anticoagulants other than drugs given therapeutically are rarely found in blood (17) However, as the spontaneously-occurring anticoagulants may be responsible for serious or even fatal *haemophilia-like symptoms, their demonstration in the laboratory must* be considered. The most common type of anticoagulant prevents the formation of blood thromboplastin but has no action on formed thromboplastin, prothrombin or thrombin Heparin or heparin-like material with an antithrombin activity, spontaneously present in blood, is met with excessively rarely

The anticoagulants have been detected in three groups of patients· in haemophilia or Christmas disease, following pregnancy, and in elderly patients, sometimes complicating a variety of other illnesses

DEMONSTRATION OF AN ANTICOAGULANT BY ADDING THE PATIENT'S PLASMA TO NORMAL BLOOD OR PLASMA

Principle. A circulating anticoagulant sufficiently potent to cause clinical symptoms is always associated with a prolonged whole-blood clotting time. *If this is so, the effect of mixing the patient's blood with* normal blood can be studied A small proportion (e g , 1 part in 10) of normal blood added to a haemophilic patient's blood will reduce the clotting time to normal In the presence of a circulating anticoagulant, on the other hand, the clotting time of the normal blood will be prolonged, rather than that of the patient shortened, in mixing tests. Similar tests can be carried out—often more conveniently—using citrated plasma from the patient and from a normal subject.

Method (6)

With Whole Blood Samples of blood from the patient and from the normal control *are collected in siliconed syringes* and each is delivered into a siliconed tube chilled in ice-water. Five 75×8-mm. coagulation tubes (not siliconed) are placed in a water-bath at 37°C. Using a siliconed pipette, 1 ml , 0 9 ml , 0·5 ml and 0·1 ml of the patient's blood are delivered into tubes *1 to 4, respectively, and, without delay,* 0 1 ml , 0·5 ml , 0 9 ml. and 1 ml of normal blood are delivered into

tubes 2 to 5, respectively. The contents of the tubes are immediately mixed by inversion and their clotting times measured in the usual way.

With Citrated Plasma. Lightly centrifuged ("low-spun") citrated plasma from the patient and from a normal control are prepared using siliconed glassware. *The two plasmas are then mixed in the same* proportions as for the whole-blood method. The tubes are placed at 37°C. and the *mixtures then recalcified by adding* an equal volume of 0·025M-*calcium chloride to each* plasma mixture. The clotting times are recorded.

The anticoagulant effect *may sometimes* be *more* definite *if* the plasma mixtures are allowed to stand for a time at 37°C. before recalcification. Therefore, *if the results of the test* are negative or indecisive, it should be repeated, *recalcifying after* 15 minutes' incubation.

Demonstration of an Anticoagulant using the Thromboplastin-Generation Test

[illegible] *n be detected in both the patient's* [illegible] n. Using either reagent, therefore, [illegible] *An immediate separation from* [illegible] ius be made, for in the former the [illegible] *latter the Al(OH)₃-treated plasma.* If the anticoagulant is present only in plasma, differentiation from haemophilia can be *made if it can* be shown that normal plasma (even in high concentration) does not correct the impaired thromboplastin generation

Demonstration of Heparin-like Activity in Plasma

Principle [illegible] fibrinogen rea[illegible] thrombin (6) [illegible] constituent o[illegible] *[illegible] plasma can be detected by demonstrating that the* [illegible]

ESTIMATION OF FIBRINOGEN (AS FIBRIN)

Principle. Oxalated plasma is clotted with calcium chloride and the fibrin preserved. This is then digested with sulphuric acid and selenium dioxide. The protein nitrogen is estimated, as ammonium sulphate, with Nessler's solution *The nitrogen figures, multiplied by* 6·25, give the approximate protein concentration.

Method (King (19))

The colour produced is compared, using a photoelectric colorimeter provided with a blue-green (Ilford 623) filter, with that produced by 5 ml. of a standard ammonium chloride solution, containing 0·01 mg. of nitrogen per ml., to which 3 ml. of Nessler's solution have been added.

Calculation

Fibrin (g. per 100 ml. plasma)

$$= \frac{\text{Reading of test}}{\text{Reading of standard}} \times 0{\cdot}05 \times \frac{100}{0{\cdot}1} \times \frac{6{\cdot}25}{1{,}000}$$

$$= \frac{\text{Reading of test}}{\text{Reading of standard}} \times 0{\cdot}3125$$

Solutions

50% (v/v) sulphuric acid containing 1% selenium oxide (Ref. 19, p. 12).
Nessler's reagent (Ref. 19, p. 10).

Normal Range of Plasma Fibrinogen: 200–400 mg. per 100 ml.

DEMONSTRATION OF EXCESSIVE FIBRINOLYSIS

Principle. Patient's diluted plasma is clotted by thrombin or calcium chloride, and incubated at 37°C. for 24 hours. Dilutions of a normal plasma are set up at the same time and lysis of the clots in the two sets of preparations looked for and compared (6). This method sets a high threshold for fibrinolytic activity but is useful clinically. Normal plasma-clots do not as a rule undergo any lysis.

An alternative and more sensitive low-temperature technique has been described (12) by which normal plasma can be shown to have fibrinolytic activity if the plasma is kept cold before the tests are set up.

Method (6)

0· ... 7·35,
to g... red:
(a) 1... l. of
buffe... ered
salin... ition
(see ... cium
chloride. The contents of the tubes are mixed by inversion and the six

Method

Rather more than 5 ml. of blood are withdrawn into a dry syringe. The blood is carefully delivered without delay into a clean test-tube, and 5 ml. are then transferred by pipette into the graduated centrifuge tube. The glass rod

a minute or so for the clot to drain, the volume of expressed serum and cells is read off directly from the graduations on the tube. *The volume expressed as a percentage of 5 ml. represents the percentage retraction of the clot.*

Normal Limits of Clot Retraction (23)

In 50 subjects (27 men and 23 women) retraction varied from 48% to 64% with a mean of 54 7% The statistical limits ($\pm 3\sigma$) were 44% to 65·5%

Significance of Clot-Retraction Measurement

The relative failure to retract of clots formed in platelet-deficient blood or plasma is well known, and the clot retraction test has been widely used as a measure of platelet function Other factors, in addition to the platelets, which affect retraction are the temperature—retraction is faster and more complete as the temperature is raised to 42°C.; the volume of red cells and leucocytes which play a passive obstructive role—there is an almost linear inverse relationship between the volume of packed cells and clot retraction, and the concentration of fibrinogen, to which clot retraction is inversely related (8).

TESTS FOR PLATELET ANTIBODIES

It now seems likely that "idiopathic" thrombocytopenic purpura may be brought about by the formation of anti-platelet antibodies (1) A number of techniques for the demonstration of the antibodies *in vitro* have been devised (13, 14, 16, 20, 21, 25, 36, 38, 39) None appears to be completely satisfactory.

It is clearly less easy to demonstrate platelet agglutinins than red-cell agglutinins There are several reasons for this: the patient's platelets on which antibody might be adsorbed—and hence demonstrable, perhaps by an antiglobulin technique—are usually present only in small numbers in the blood of the patient and are thus difficult to work with; antibody free in the serum seems usually to be present, if at all, in such small amounts that minor degrees of agglutination have to be accepted as being significant; intra-platelet-group antibodies may be a source of confusion, finally, agglutination of platelets is less easy to see than agglutination of red cells.

volume of 0 33N-citric acid

Harrington (15) stresses that as platelets vary in their sensitivity to

final platelet concentration

Tullis (39) added preserved human platelets and diluted normal serum (as a source of complement) to the test serum which had been previously adsorbed with barium sulphate The platelet suspension is incubated at

volumes of the patient's plasma and the platelet-rich normal plasma are

exchange resin (IRC-50)

More recently, Malinvaud and Dausset (25) have recommended that the suspension of platelets in the patient's serum be agitated for 30 minutes at room temperature using a shaker designed for the Kline test (110 movements per minute).

unproven (1) Finally, attempts have been made to demonstrate antibodies

adsorbed to platelets by means of the antiglobulin reaction Although [illegible]

strate the corresponding drug-induced agglutinating and lytic platelet antibodies.

TABLE XIV

INVESTIGATION OF A HAEMORRHAGIC DISORDER

Patient's name	No
Date	Diagnosis

1	Haemoglobin (g /100 ml)	Blood film
2	Platelet count (/ c mm)	Platelet morphology
3	Whole-blood coagulation time (min) (Lee-White, 37°C)	
3a	Bleeding time (min) (Duke)	
4a	One-stage prothrombin time (human brain) (sec)	
	+ 1/10 vol fresh normal plasma (sec)	
	+ 1/10 vol normal serum (sec)	
4b	Prothrombin-consumption test	
	1 min plasma time (sec)	
	1 min. serum time (sec)	
	Prothrombin-consumption index (P C I) (%)	

TABLE XIV (*continued*)

4c Thromboplastin-generation test	Incubation time (min.)									
	1	2	3	4	5	6	7	8	9	10
	Clotting times (sec.)									
Patient's plasma Patient's serum Normal platelets										
Normal plasma Patient's serum Normal platelets										
Patient's plasma Normal serum Normal platelets										
Normal plasma Normal serum Normal platelets										
Normal plasma Normal serum Patient's platelets										
4d Tests for anticoagulants										
4e Thrombin-fibrinogen reaction time (sec.)										
4f Fibrinogen (mg./100 ml.)										
5a Clot retraction (%) (Macfarlane, 37° C.)										
Capillary resistance test (Hess test)										
Other tests										
Conclusion										

PRACTICAL ASPECTS OF THE LABORATORY DIAGNOSIS OF A HAEMORRHAGIC DISORDER

In the following section a scheme is suggested for the laboratory investigation of a patient suspected of suffering from a haemorrhagic disorder. The recommended procedures are given in italics.

1. *Haemoglobin estimation and inspection of a stained blood film* The diagnosis may be obvious at this stage, as in leukaemia.
2. *Platelet count.*
3. *Whole-blood clotting time, bleeding time and capillary resistance test.* In most instances it should be possible by this stage to have decided whether the patient is suffering from (*a*) a coagulation or (*b*) a platelet and/or vascular defect. (The personal and family history of the patient and the clinical findings may also be of help in deciding to which category the patient belongs.)
4. If a coagulation defect is suspected, proceed as follows:
   - 4a. *One-stage "prothrombin" time.* If prolonged, carry out: (I) qualitative tests for Factor V and Factor VII; (II) two-stage estimation of prothrombin.
   - 4b. *Prothrombin-consumption test*
   - 4c *Thromboplastin-generation test.* This should enable a [illegible] (Table XIII)
   - 4d *Tests for circulating anticoagulants.* If, using the thromboplastin-generation test, a defect is demonstrated in the patient's serum as well as in his plasma, the presence of a circulating anticoagulant is possible Try the effect of adding normal plasma or serum to the patient's plasma or serum preparations used in the thromboplastin-generation test, and carry out other tests for circulating anticoagulants and for heparin-like substances
   - 4e *Tests for abnormal fibrinolysis and thrombin-fibrinogen reaction time*
   - 4f. *Estimation of plasma fibrinogen*
5. If a platelet and/or capillary defect is suspected proceed as follows:
   - 5a *Clot retraction test and prothrombin-consumption test*
   - 5b *Test for platelet function in the thromboplastin-generation test.* Compare the relative potency of preparations of normal platelets and the patient's platelets.
   - 5c. *Tests for platelet antibodies.*

The most useful tests for routine diagnostic purposes are those numbered 1, 2, 3, 4a and 4c. A suitable laboratory work sheet for the recording of the results of the tests is illustrated in Table XIV.

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IgM – is Complete Antibodies. Because it ... and it agglutinates in Saline ... → Complete Ant...
IgG → Incomplete Antibodies ! because ... (For ... and at 16°, ... and do not agglutinate ... suspension

CHAPTER II

BLOOD GROUPS AND THE LABORATORY ASPECTS OF BLOOD TRANSFUSION

No attempt will be made to give a detailed survey of the human blood groups. In this chapter will be described only some essential basic facts and selected practical techniques useful in the management of a hospital blood ...

graph of Race and Sanger (26)

THE HUMAN BLOOD GROUPS

It is now realized that human red cells contain far more inheritable blood-group antigens than was at one time thought At least nine well defined systems of antigens are recognized at the present time; these are, in order of discovery, the ABO, MNSs, P, Rh, Lutheran, Kell, Lewis, Duffy and Kidd blood-group systems Fortunately, only the ABO and Rh systems are of major clinical importance, the others are of less importance because the corresponding antibodies are either not present normally or occur only rarely, and, because, when present, they usually react only at low temperatures

THE ABO BLOOD-GROUP SYSTEM

There are only four main groups, i e , AB, A, B, and O, but sub-groups of A and AB also exist Slightly over 20% of group-"A" and "AB" subjects belong to group A_2 and A_2B, respectively, the remainder belong to group A_1 and A_1B

The serum of a group-O person normally contains antibodies (anti-A and anti-B) which are absorbed by, and cause the agglutination of, both A and B cells, that of a group-A person contains anti-B, that of a group-B person anti-A, and that of a group-AB person neither anti-A nor anti-B (Table XV) Anti-A and anti-B are not normally present in the cord blood of newborn infants, but are formed in the first 3 to 6 months of post-natal life For this reason it is not quite clear whether they should be looked upon as naturally-occurring. Anti-A and anti-B, although active at 37°C, normally cause more intense agglutination and act in higher dilutions at temperatures below 37°C. Their temperature characteristics are thus intermediate between

those of " warm " antibodies such as anti-Rh and non-specific " cold " antibodies (see p. 203).

TABLE XV

THE ABO ANTIGENS AND AGGLUTININS

Blood group	Agglutinins normally present in serum	Agglutinins occasionally present in serum
A_1B	none	anti-H (α_2) (rare)
A_2B	none	α_1 (25–30% of sera)
A_1	anti-B (β)	anti-H (α_1) (rare)
A_2	anti-B (β)	α_1 (1–2% of sera)
B	anti-A ($\alpha + \alpha_1$)	anti-H (α_2) (rare)
O	anti-A ($\alpha + \alpha_1$) + anti-B (β)	none

Subgroups of A

Anti-A sera from group-B subjects nearly always contain two antibodies acting on group-A red cells, α and α_1. α agglutinates A_1, A_2, A_1B and A_2B cells, but α_1 only agglutinates A_1 and A_1B cells. The net result is that group-A_2 and -A_2B cells are less strongly agglutinated by anti-A sera than are group-A_1 and -A_1B cells. Still weaker reacting forms of A exist, e g , A_3 and A_4, but these appear to be very rare. Intermediate forms between A_1 and A_2 are also thought to occur.

The existence of the subgroups of group A has a practical bearing, for no serum can be deemed satisfactory for blood-grouping until it has been shown to give strong reactions with the relatively weakly reacting group-A_2 blood.

Antibody reacting only with A_1 and A_1B cells (α_1) occasionally occurs

Immune Types of Anti-A and Anti-B

very sensitive paroxysmal nocturnal haemoglobinuria (P.N H) red cells is accepted as a reliable criterion (8). Some human sera, however, are undoubtedly much more active in haemolysing normal red cells than others of similar agglutinin content.

The strongly haemolytic sera are commonly referred to as "immune"

Methods of ABO Grouping

Principle. The red cells are tested for their agglutinability at room temperature by sera containing highly potent anti-A and anti-B, respectively. The agglutination can be carried out on tiles or in tubes

The Tile Technique

Agglutination is rapid on flat or slightly concave surfaces, and the method is particularly useful when only a few samples of blood are to be grouped

One drop of each grouping serum and one drop of saline are delivered to two of the concavities on a white porcelain tile, the concavities are labelled, respectively, "anti-A" and "anti-B" One drop of an approximate 5% suspension of the red cells to be tested is added to each grouping serum and the cell suspensions then mixed by gently rocking the tile. After 5 minutes or less the results may be read; they are usually clear-cut and indisputable If doubtful, the presence or absence of agglutination may be checked by transferring the suspension on to a glass slide and viewing under the low powers of a microscope

Controls. It is important to set up controls using known group-A_2, -B and -O red cells The group-A_2 and -B cells test the sensitivity of the grouping sera and the O red cells, which should not be agglutinated, guard against the possibility of false positive reactions (see later) It is essential to have available agglutinating sera capable of agglutinating A_2 cells distinctly within a few minutes, if the sera are sufficiently potent, it is an advantage, as recommended above, to add one volume of saline to the grouping serum on the tile so as to minimize rouleaux formation

The tile method is sensitive and accurate, but agglutination of the very rare weakly reacting forms of group A, such as A_3, may be missed

Room temperature (15° to 25°C.) rather than 37°C. is chosen as the temperature for the test, as this is the optimum for the normal agglutinating type of anti-A or anti-B.

The Tube Technique

The tube method is slower than the tile technique, but it is more suitable for the grouping of large numbers of samples. The results can be *read macroscopically or microscopically*. It is thus better adapted than is the tile technique for detecting the rare weakly reacting cells

The tests can be carried out in 50×6-mm. or 75×8-mm. serological *tubes*. One drop of each grouping serum is added to two tubes labelled anti-A and anti-B, respectively. One drop of saline is added to each tube and then one drop of a 1 to 2% suspension of the red cells to be tested. The suspensions are mixed by tapping the tubes which are then left undisturbed for 2 hours at room temperature. The presence or absence of agglutination can be determined by inspection of the button of deposited cells using a concave mirror, followed by gentle resuspension Doubtful reactions should be assessed by transferring the cell-serum suspension to a glass slide and viewing it under the low powers of the microscope. As a rule, if the agglutinating sera are avid, i e., cause rapid intense agglutination, the reactions are absolutely clear-cut and can be read macroscopically with confidence.

Controls It is essential, as in the tile technique, to set up controls, using known group-A_1, -B and -O blood

CHECKING THE ABO GROUP BY TESTING FOR ANTI-A AND ANTI-B

[illegible]

subgroups of A

Selection of Grouping Sera

[illegible]

clot at 37°C. and then, after the serum has started to separate, placed in a refrigerator overnight for the clot to retract, and for any cold agglutinins present to be at least partially absorbed The serum is then centrifuged to free it from suspended cells, inactivated at 56°C. for 30 minutes, Seitz-filtered and finally bottled in 1 to 2-ml volumes The sterile sera should be kept frozen at −20°C. until used. It is essential *to keep the sera free from bacterial growth, which may cause non-specific* "bacteriogenic" agglutination (9).

It is usually possible, in transfusion centres where large numbers of samples are available for testing, to select avid grouping sera with a titre sufficiently

high for routine purposes. Alternatively, the agglutinin titre of a serum may be raised significantly by the subcutaneous or intravenous injection of commercially prepared A or B substances. Rises in titre of 2 to 64 times have been recorded (27) Such sera also develop a strong avidity for the corresponding red cells and cause very rapid agglutination in tile tests (32).

Causes of False-Positive Reactions in ABO Grouping

In addition to the bacteriogenic agglutination mentioned above, there are several other possible causes of falsely positive agglutination: rouleaux formation simulating true agglutination, agglutination by non-specific cold agglutinins, and infected red cells

Rouleaux Formation

This should rarely be a source of trouble No serum which causes rouleaux with normal cells should be used for grouping, and the volume of saline added to the serum serves as an extra precaution. However, in dealing with red cells from a patient whose serum contains high concentrations of abnormal globulins, as in multiple myeloma, sufficient patient's serum might be carried over in the red-cell suspension to cause marked rouleaux. It is important, therefore, in such a case to wash the patient's cells thoroughly in saline before attempting to group them.

Cold Agglutinins

These may be also a cause of trouble If the patient's serum contains agglutinins active at room temperature, it is imperative in order to obtain a smooth suspension that his red cells be carefully washed in several changes of saline warmed to 37°C before they are added to the grouping serum In acquired haemolytic anaemia caused by warm auto-antibodies, highly sensitized cells reacting strongly with antiglobulin serum may undergo auto-agglutination in normal serum, even after thorough washing However, the presence of saline in the grouping test should in most cases be sufficient to prevent this. In doubt, tests for anti-A and anti-B in the patient's serum should help to clear up any difficulty

The grouping sera themselves must not contain any non-specific cold agglutinin active at the temperature of the laboratory.

Infected Red Cells

Infection of a red-cell suspension by certain bacteria may result in the cells becoming agglutinable by normal adult human sera* (Hubener-Thomsen-Freidenreich phenomenon) Such cells might appear to be group AB when in reality they were group O In view of this possibility groupings should be carried out as soon as possible after the blood has been collected or, if delay is unavoidable, the blood

* Infection *in vivo* is thought to be the cause of the rare phenomenon of polyagglutinability Polyagglutinable red cells undergo agglutination in most human sera except that of young infants (26).

should always be kept at 4°C. If sterility cannot be guaranteed blood is best stored as a clot rather than as blood to which an anticoagulant has been added. On no account should red cells be stored as a saline suspension.

Causes of False-Negative Reactions in ABO Grouping

Failure of agglutination to take place is usually due to impotent sera. Loss of potency results if sera are carelessly left at room temperature or stored frozen in large volumes so that repeated freezings and thawings are required. Another possible cause of a false-negative reaction is the use of fresh rather than heat-inactivated sera. This may lead to rapid haemolysis being mistaken for absence of agglutination.

Differentiation of Group A_1 from Group A_2

Principle. Anti-A serum from a group-B subject contains two antibodies, α and α_1. If such a serum is absorbed with A_2 cells, α is removed; α_1 remains. The absorbed (α_1) serum then reacts only with A_1 cells

Method

1 volume of anti-A serum is added to an equal volume of washed packed A_2 cells. The cells are mixed in the serum and the suspension allowed to stand undisturbed at 4°C for 1 hour. It is then centrifuged and the supernatant serum tested for its power of agglutinating known A_1 and A_2 cells The A_1 cells should still be agglutinated (although less strongly than before because of the removal of α); the A_2 cells should not be agglutinated at all. If they are agglutinated the absorption should be repeated. *The reactions are usually clear-cut* However, cells which give weak reactions with the absorbed sera may be encountered, these may possibly belong to a type "intermediate" between A_1 and A_2 (31)

Group-A_1 and -A_2 cells can also be differentiated in other ways [illegible]

Tests for Secretion of A or B Substance

[illegible] to anti-A or anti-B serum [illegible] the antibodies have been [illegible] ors (they are Le^a positive, [illegible] ance, or H substance (if group O)

Method

An anti-A or anti-B serum is diluted so that it gives good visible agglutina-

hours and are then inspected for agglutination If the saliva contains A or B substances, agglutination is usually inhibited in all the tubes except the saline-control tube.

H substance can be demonstrated in a similar way using eel serum or the naturally occurring incomplete cold antibody as a source of anti-H (6).

TITRATION OF ANTI-A OR ANTI-B AGGLUTININS

Principle. Serial dilutions of the sera are made in saline, and equal volumes of a saline suspension of washed group-A_1, -A_2 or -B red cells added. Agglutination is read after the suspensions have been allowed to stand for 2 hours at room temperature

Method

ml volumes of serum or saline are suitable for use with 75×8-mm. tubes It is convenient to retain the surplus volume of diluted serum from tube No 9 (which otherwise would be discarded) so that if the serum is found to contain very high-titre agglutinins further dilutions may be made

The red cells used for the titration should be freshly withdrawn or obtained from blood which has been stored in ACD at 4°C for not more than a few days

After washing in two changes of saline, a 1 % suspension is prepared An equal volume of this 1 % suspension is added to each of the serum dilutions and to the saline-control tube After thorough mixing, the suspensions are allowed to stand undisturbed at room temperature (15 to 25°C) for 2 hours

Agglutination is then looked for macroscopically or microscopically, as described on p 106

The *agglutinin titre* is usually recorded (by blood-group serologists) as the reciprocal of the highest *serum* dilution which gives ± agglutination This figure does not take into account the volume of red cells added.

Either A_1 or A_2 cells can be used. In the selecting of a serum for routine blood grouping, its reaction with A_2 cells is particularly important. Hence, there is some advantage in using A_2 cells for titration of anti-A.

Demonstration of Haemolysis by Anti-A or Anti-B

Principle. Group-A (or -B) cells are suspended in fresh undiluted anti-A or (anti-B) serum or in serum serially diluted in a source of complement. Haemolysis is looked for after incubation for 2 hours at 37°C.

Method

Qualitative Test. One volume of a 50% suspension of washed group-A_1 (or -B) red cells is suspended in nine volumes of fresh anti-A (or -B) serum The mixture is incubated at 37°C. for 2 hours, then centrifuged and the supernatant inspected for haemolysis. The serum should be tested within a day or so of collection.

Titration of Haemolysin

... of the same

inspected for haemolysis

... has to be of the same

patient's serum) is essential.

Demonstration of Incomplete Anti-A or Anti-B

... either saliva

when serum is used as a diluent but not when saline is used.

Method Using Saliva (5)

... and to
e-half
the
one-
tube.

After 2 hours the cells are washed in several changes of saline and an antiglobulin test is carried out. The antiglobulin serum should be used at a dilution not exceeding 1 in 4

THE RH BLOOD GROUPS

The Rh Antigens

There are thought to be at least three systems of allelomorphic genes situated close together in the same chromosome The common alternative antigens to which they give rise are, in Fisher's notation, C or c, D or d, and E or e Recently a further system, F and f has been postulated Less common alternative antigens are C^w, C^u, C^v, and C^x, D^u, E^u and E^w. The large number of antigens involved means that many different combinations are possible. Leaving aside the less well known or rarer variants the common alternative genes for *C*, *c* and C^w, *D* and *d*, and *E* and *e* can be combined in a chromosome in twelve ways These are in the order of their frequency *CDe*, *cde*, *cDE*, *cDe*, C^wDe, *cdE*, *Cde*, *CDE*, C^wde, *CdE*, C^wDE and C^wdE In pairs these [illegible]

For clinical purposes the combination *cde/cde* is called Rh-negative (D-negative), all the rest are Rh-positive (D-positive) The commonly used short symbols corresponding with the common and less rare chromosomes are given in Table XVI.

TABLE XVI

THE RH CHROMOSOMES IN ORDER OF FREQUENCY (FISHER NOMENCLATURE) AND THE CORRESPONDING SHORT NOTATIONS (26)

Fisher	Short notation
CDe	R_1
cde	r
cDE	R_2
cDe	R_0
C^wDe	R^w_1
cdE	R″
Cde	R′
CDE	R_z
CdE	R_y

PRACTICAL IMPORTANCE OF RH ANTIGENS

The Rh antigens are of practical importance because, although the corresponding antibodies, with rare exceptions (13), do not occ naturally, they not uncommonly develop (*a*) as the result of dif

in the Rh blood-group antigens of a foetus and its mother, and (*b*) as the result of transfusion of blood containing Rh antigens which the recipient lacks. Anti-D is most frequently formed, but many examples of anti-E and anti-C, anti-c and anti-C^w, and anti-e are known.

Rh Grouping of Transfusion Recipients

Before any patient is transfused, it is essential to determine whether he is D-positive or D-negative and to give him blood of the corresponding type, i.e., D-positive or D-negative, respectively. It is not necessary for most purposes, nor would it be practical, to find what his other Rh antigens are Even if a patient's genotype were accurately known, it would in most instances not be easy to find blood of exactly the same genotype, even if the matching was confined to the ABO and Rh groups In transfusion work the best that can be done as a routine is to give the recipient D-positive or D-negative blood, as required, and to carry out a careful cross-matching test to guard against the presence of antibodies such as anti-E or anti-c which might have been developed as the result of past pregnancies or apparently compatible transfusions (see also p. 201).

However, there are certain patients in whom it is important to know the Rh genotype: e.g., in patients suffering from acquired haemolytic anaemia when it is desirable to try to identify the specificity (if any) of the auto-antibodies; and in patients who need repeated transfusions—it is desirable to transfuse such patients with blood of the same Rh genotype as far as is practicable Although they are not candidates for transfusion, the husbands of women who have given birth to infants suffering from haemolytic disease of the newborn, should have their blood genotyped, for, in order to give a prognosis of future pregnancies, it is essential to know whether the husband is heterozygous or homozygous for the immunizing antigen

Rh-Grouping of Transfusion Donors

When grouping blood donors for their Rh groups it is essential to test D-negative red cells against anti-E and anti-C sera as well, to indentify the small proportion who are of the genotype *cdE/cde* or *Cde/cde* and whose blood should not be used to transfuse D-negative recipients In practice, in order to conserve rare sera it is convenient to use the more easily obtained anti-D + anti-C and anti-D + anti-E sera in place of pure anti-C or pure anti-E, and to test only those cells which fail to react with anti-D If the genotypes *cdE/cde* and *Cde/cde* [illegible]

with an incomplete anti-D known to react with D^u cells (24)

METHODS OF Rh GROUPING

Three techniques will be described: tube methods using (*a*) a complete (in-saline-agglutinating) anti-D or (*b*) an incomplete (in-albumin-agglutinating) anti-D, and Chown's capillary-tube method (3, 4)

Tube Method Using Saline Anti-D

It is desirable to use small 50 × 6-mm. tubes in order to economize with the grouping serum. One small drop (0·02 ml. approximately) of grouping serum is carefully dropped to the bottom of three serological tubes, and one drop of a 2% suspension of the test red cells and known D-positive and D-negative cells is added to the three tubes, respectively. After making sure that the cells are well mixed with the serum, the tubes are covered with glass caps and left undisturbed for 2 hours in an incubator at 37°C. The presence or absence of agglutination may be ascertained by viewing the button of sedimented cells with a concave mirror—a negative result is shown by a perfectly smooth-edged small round button and positive agglutination by a larger deposit of cells with irregular or fluffy edges—and then confirming the result microscopically. The deposited cells are examined under the low powers of the microscope after carefully transferring a little of the deposit on to a slide and gently spreading it out with the drawn-out stem of a Pasteur pipette.

As it is essential for the ready recognition of agglutination to view the cells in a weak suspension, about a 10-mm column of supernatant serum should first be taken up into the Pasteur pipette before its tip (which must be cut off transversely) is dipped into the cell deposit and a very small column of cells withdrawn into it. As the clumps of agglutinated cells are easily broken up, at least using sera of low titre, care and experience are necessary for the interpretation of results. A temperature of 37°C is about optimal for agglutination.

As a rule the reactions are absolutely clear-cut if a potent anti-D serum is available. A weak or doubtful reaction—when the control D-positive cells are strongly agglutinated—may be due to D^u. This should be checked by seeing whether the doubtfully agglutinated cells are agglutinated by antiglobulin serum (which they should be if they are in fact D^u).

The Grouping Serum

This should be a pure anti-D serum, its titre, if possible, exceeding 16. If it agglutinates D^u cells, so much the better. The serum may have to be absorbed with A_1 or B, Rh-negative cells, if anti-A or B is present. If both antibodies are present, washed packed A_1B, Rh-negative cells may be used for the absorption. Absorptions are best carried out at 4°C. Alternatively, saliva from secretors of A and/or B can be used, but this has the disadvantage of entailing some dilution of the serum

Tube Method Using Incomplete Anti-D

Exactly the same technique is used as with the saline anti-D, except that an additional volume (one small drop) of 30% bovine albumin is added to each tube. The tubes are incubated for 2 hours at 37°C. and the presence or absence of agglutination is then determined microscopically.

An alternative technique for use with incomplete anti-D has recently been described by Low (20a). In Low's method a solution of activated papain is substituted for the albumin. Agglutination of D-positive red cells takes place relatively rapidly (see also p. 121).

Storage of Rh Grouping Sera

Anti-D sera are best kept frozen at −20°C in small volumes (0.5 to 2 ml). They may be kept at this temperature for several years without undergoing much deterioration. However, if kept unfrozen at 4°C in a refrigerator, they lose their potency more rapidly. If sera have to be kept unfrozen, it is advisable to add a bacteriostatic agent such as 0.1% sodium azide. The addition of bovine albumin may also help to maintain their potency (16).

Chown's Capillary-Tube Method (3, 4)

Small glass capillary tubes of about 0.5 to 1.0-mm. bore and about 90 mm. in length are used, and enough "saline" anti-D serum allowed to run in by capillarity to give a column of approximately 20 to 30 mm. The tube is then dipped into a 20 to 30% saline suspension of washed test cells and a column of cells of about the same length as that of the serum allowed to run up the tube in contact with the serum. The opposite end of the tube is sealed in a flame and it is then stood up in Plasticine at an angle of about 45°, the cell suspension being uppermost. The sealed capillary can, with advantage, be placed in a specially designed, inclined rack which is illuminated from behind.

The capillary tube or rack of tubes is allowed to stand undisturbed at room temperature—warmth is desirable but not essential. The cells will gradually sediment through the serum. If D-negative, they will roll down the inferior surface of the lumen of the tube and eventually form a solid column at the bottom, if D-positive, they form irregular clumps which fail to fall to the bottom of the tube, leaving instead an irregular beaded layer of clumped cells along the length of the tube. At an early stage, before sedimentation has been completed, agglutination gives a characteristic irregu[illegible] to the column of sedimenting cells, in the ab[illegible] of aggl[illegible]e margins are smooth and straight.

The resu[illegible] ithin 15 minutes, agglutina-
tion may o[illegible] are usu[illegible] there is any doubt, the tube
should be i[illegible] much [illegible] ce more. In
addition, a t[illegible] c cells [illegible] l tube
[illegible] t up

Relative Merits of the Serological Tube Technique and Chown's Capillary-Tube Method

Probably the best and most reliable method of Rh grouping is the rological-tube method, using a potent saline anti-D serum. The :sults are usually more definite and less experience is required in their iterpretation than when an incomplete antibody plus albumin is used. [he difficulty with the albumin method is the frequency with which D-negative cells show slight rouleaux formation or minor degrees of sticking together. Both methods may need at least 1 hour for unmistakable agglutination to develop, unless unusually potent anti-D sera are available. Neither is suitable for rapid Rh grouping.

Chown's capillary-tube method has certain definite advantages; in particular, it is economical of serum and agglutination takes place rapidly. However, a potent anti-D serum is required, and the possibility of false positive results due to rouleaux formation exists It is therefore imperative to wash the test cells before use, and if the grouping serum is sufficiently potent to withstand dilution with saline so much the better. In the author's opinion Chown's method is the most reliable available technique if the result is wanted quickly With a good grouping serum it is often possible to detect a positive reaction within 5 minutes.

Rh Genotyping

Determination of the probable Rh genotype has usually to be left to a specialist laboratory where the necessary sera are available. The following sera are required anti-D, anti-C, anti-C^w, anti-E, anti-c, and anti-e As many of the sera are rare and precious, the volumes used must be kept to a minimum A Pasteur pipette, graduated to deliver about 0 01 ml is used The serum is placed at or very near to the bottom of a 50 × 6-mm tube and the red cells a little higher up, care being taken not to touch the bottom of the tube which has been wetted by the serum (26) The red cells are then shaken down into the serum and mixed in it The tubes are conveniently set in wooden blocks containing 50 holes, 10 mm in diameter, arranged in 5 rows of 10 holes Ideally, in-saline-agglutinating sera should be used, if sera containing incomplete antibodies have to be used, one volume of 30% albumin must be added in addition, or the indirect antiglobulin technique employed It is essential to use a panel of cells of known genotypes as controls

A good deal of information can be obtained by the use of *four* relatively easily available sera anti-C + C^w, anti-c, anti-D and anti-E This will enable the genotype to be determined with a fair degree of probability For instance, if the reactions with all four sera are positive, the probable genotype is *C De/cDE* Although there are eleven other rarer possible combinations, the next commonest possibility *CDe/cdE* is about 12 times less frequent than is *CDe/cDE* If pure anti-c,

pure anti-C^w, and anti-e are also available, the extra possibilities are reduced to five (26). A knowledge of the reactions of the blood of other members of the family may help in excluding some of the possible combinations

Detection of Rh Antibodies

Several methods are available for the detection and titration of Rh antibodies For the detection of antibodies as in the routine examination of the sera of antenatal patients, there is a choice of two reliable techniques· (*a*) the indirect antiglobulin method and (*b*) a method employing enzyme-treated red cells Both methods are suitable for the detection of the rarer Rh antibodies as well as anti-D

Indirect Antiglobulin Method

To 5 or 10 drops of the patient's serum is added 1 drop of a 30 to 50% suspension in saline of Rh-positive cells The mixture is incubated at 37°C. for 2 hours and the cells are then tested for their agglutinability by antiglobulin serum, as described on p. 108. The antiglobulin serum must be diluted to its point of maximum avidity (usually 1 in 16 to 1 in 64).

As a screening test, it is best to use a sample of group-O *CDe/cDE* red cells (or separately, group-O *CDe/CDe* and group-O *cDE/cDE* cells) which have the necessary antigens to detect anti-C, anti-c, anti-E and anti-e as well as anti-D. As controls, the same red cells should be added to normal serum and to a serum containing a small amount of anti-D

If positive agglutination is obtained, it must not be assumed that this is necessarily due to anti-D (or for that matter an antibody within the Rh system), in both antenatal and transfusion work anti-E and anti-C are not infrequently found, usually in combination with anti-D, but occasionally by themselves Anti-c (by itself) is also not very uncommon Prelim[illegible]y identification of an antibody can often be effected by te[illegible] a positive reaction separately with gro[illegible] cells However, the speci[illegible] anti-D plus anti-E can onl[illegible] *Cde/cde*, *cDe/cde* and *cdE/*[illegible] [illegible]able, and special [illegible] very rare genotypes [illegible] panels of cells are, of course, necessary to identify antibodies such as anti-C^w

Method Using Enzyme-Treated Red Cells (18, 25)

The chosen test cells (e g, group O *CDe/cDE*) may be acted upon by either trypsin or papain (see p 120) One volume (2 or 3 drops) of a 2% suspension in saline of the treated cells is added to an equal volume of serum placed in a tube set in a water-bath at 37°C The tubes are examined for agglutination, using a concave mirror, after 2 hours at 37°C. It is essential to set up controls of normal serum and serum known to contain a small amount of anti-D, it is also worthwhile setting up, as additional controls, duplicate tubes to which enzyme-treated Rh-negative cells are added The test must be carefully carried

Löw's method of using papain (20a), which obviates the necessity of pre-treatment of the test cells with the enzyme, is referred to on p. 194.

Titration of Rh Antibodies

Rh antibodies may be titrated in several ways (*a*) using dilutions of the serum in saline and normal red cells, (*b*) using dilutions of the serum in saline and enzyme-treated red cells; (*c*) using dilutions of the serum in normal human group-AB (or compatible) serum and normal red cells suspended in 20% bovine albumin (The preparation of the cell suspensions is described on p 102.)

Titration in Saline

Doubling dilutions of the serum are made in saline using a marked Pasteur pipette calibrated to deliver about 0 05-ml volumes It is convenient to use 50×6-mm serological tubes and to set them in a wooden block Nine tubes, giving a range of serum dilutions from 1 in 1 to 1 in 256, are generally sufficient; a tenth tube to which saline alone is added serves as a control 0 05 ml. of a 1% saline suspension of the selected red cells (e g , group O *CDe/cDE*) is added to each tube* After gently tapping the tubes to mix the cells in the serum, the block of tubes is placed in the incubator at 37°C. and left undisturbed for

the presence of incomplete (blocking) antibody may be suspected

More often than not anti-D is present in the incomplete form and little or no agglutination takes place even in the first tube Incomplete

each tube, re-incubating for 30 minutes and examining for agglutination However, it is usually more convenient to set up at the start a duplicate titration using either trypsinized cells or cells suspended in albumin.

Titration in Saline Using Trypsinized (or Papainized) Cells

Exactly the same procedure is followed as that described in the previous section except that enzyme-treated red cells are substituted

* If more than one type of cell is to be tested it is advisable and more accurate to make primary dilutions of the serum in 75×8-mm tubes, and to deliver 0 02 or 0 05-ml subsamples of each dilution into small serological tubes

pure anti-C^w, and anti-e are also available, the extra possibilities are reduced to five (26). A knowledge of the reactions of the blood of other members of the family may help in excluding some of the possible combinations

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Several methods are available for the detection and titration of Rh antibodies For the detection of antibodies as in the routine examination of the sera of antenatal patients, there is a choice of two reliable techniques: (*a*) the indirect antiglobulin method and (*b*) a method employing enzyme-treated red cells. Both methods are suitable for the detection of the rarer Rh antibodies as well as anti-D.

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and anti-e as well as anti-D.
added to normal serum and
anti-D

If positive agglutination is obtained, it must not be assumed that this is necessarily due to anti-D (or for that matter an antibody within the Rh system), in both antenatal and transfusion work anti-E and anti-C are not infrequently found, usually in combination with anti-D, but occasionally by themselves Anti-c (by itself) is also not very uncommon Preliminary

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out at 37°C, for many sera contain sufficient non-specific cold agglutinin to cause agglutination of enzyme-treated red cells at room temperature. [illegible]

Löw's method of using papain (20a), which obviates the necessity of pre-treatment of the test cells with the enzyme, is referred to on p. 194.

TITRATION OF RH ANTIBODIES

Rh antibodies may be titrated in several ways. (*a*) using dilutions of the serum in saline and normal red cells, (*b*) using dilutions of the serum in saline and enzyme-treated red cells, (*c*) using dilutions of the serum in normal human group-AB (or compatible) serum and normal red cells suspended in 20% bovine albumin. (The preparation of the cell suspensions is described on p 102.)

Titration in Saline

Doubling dilutions of the serum are made in saline using a marked Pasteur pipette calibrated to deliver about 0 05-ml volumes It is convenient to use 50×6-mm serological tubes and to set them in a wooden block. Nine tubes, giving a range of serum dilutions from 1 in 1 to 1 in 256, are generally sufficient, a tenth tube to which saline alone is added serves as a control 0 05 ml. of a 1% saline suspension of the selected red cells (e g, group O *CDe/cDE*) is added to each tube*. After gently tapping the tubes to mix the cells in the serum, the block of tubes is placed in the incubator at 37°C and left undisturbed for 2 hours. The presence or absence of agglutination is then determined microscopically, as described on p 193 Occasionally, agglutination may be partially or completely inhibited in the first 2 or 3 tubes, if so the presence of incomplete (blocking) antibody may be suspected

More often than not anti-D is present in the incomplete form and little or no agglutination takes place even in the first tube Incomplete [illegible] each tube, re-incubating for 30 minutes and examining for agglutination However, it is usually more convenient to set up at the start a duplicate titration using either trypsinized cells or cells suspended in albumin.

Titration in Saline Using Trypsinized (or Papainized) Cells

Exactly the same procedure is followed as that described in the previous section except that enzyme-treated red cells are substituted

* If more than one type of cell is to be tested it is advisable and more accurate to make primary dilutions of the serum in 75×8-mm tubes, and to deliver 0 02 or 0 05-ml subsamples of each dilution into small serological tubes

for the normal cells. However, care must be taken to ensure that the test is carried out strictly at 37°C
can usually
the result is
specific aggl
If normal human serum is used as diluent instead of saline, the agglutinin titre is usually enhanced (15). Löw's method (20a) using papain activated by cysteine is referred to on p. 194.

Titration in Serum-Albumin

Serial doubling dilutions of the serum are made in normal group-AB serum; if group-O cells are used, normal serum of any ABO group will do. The control tube contains normal serum only. An equal volume of a 1% suspension in 20% albumin (see p. 103) of the selected test cells (e g., group O *CDe/cDE*) is added to each serum dilution and to the normal serum *in the control tube, and after ensuring that the cell suspension* and serum dilutions are well mixed, the tubes are allowed to stand undisturbed for 2 hours at 37°C. Agglutination is determined

should prevent mistaking rouleaux for true agglutination.

As well as being used as a diluent for the test serum, normal serum can be used as a substitute for the albumin. The method, however, seems to be less sensitive than the serum plus albumin technique It has been claimed that the sensitivity can be increased by adjusting the pH of the normal serum used as diluent to approximately pH 7 0 (2)

Relative Value of the Methods Using Albumin and Enzyme-Treated Cells

The method using enzyme-treated cells gives tighter and more definite agglutination than the serum-albumin method The end-point is also easier to read, and the agglutinin titres are likely to be higher than with albumin. Occasionally, too, methods using enzyme-treated cells demonstrate the presence of antibodies that fail to cause agglutination in serum-albumin media Prozones resulting in failure of agglutination in high concentrations of serum seem more likely to occur in the albumin method On the other hand, as has been mentioned, the sensitivity of enzyme-treated cells, particularly their agglutination by non-specific cold antibodies at relatively high temperatures, is a drawback to their use

Alternatives to Albumin in Rh-Antibody Titrations

Various substitutes for albumin have been suggested from time to time' e.g., gelatin (12), Dextran (14), and polyvinylpyrrolidone (PVP) (21) Although the above media have an advantage over albumin in sensitivity,

their value is diminished by the increased tendency to cause non-specific agglutination and rouleaux formation

Detection of Rh Antibody When Present in Very Small Amounts

Small amounts of anti-Rh can be detected reliably by the indirect anti-[illegible]

[illegible]

it is claimed, increases the agglutinating titre of the anti-D to a greater extent than does normal serum Another very sensitive technique is to carry out antiglobulin tests on trypsinized cells (29) This method needs very careful controlling using normal sera and cells negative for the antigens being sought.

THE REMAINING BLOOD-GROUP SYSTEMS

Blood grouping for the MNSs, P, Lutheran, Kell, Lewis, Duffy and Kidd antigens, and the detection of the corresponding antibodies, follows the general lines described in the foregoing discussion on anti-Rh. Tube techniques using small volumes of sera are generally employed, for the antibodies are rarely available at high titres and supplies are usually precious. The effect of trypsin on the different antigens has been studied by Morton and Pickles (25) and Unger and Katz (30) Brief notes on each blood-group system follow A full description is to be found in Race and Sanger's monograph (26)

THE MNSs BLOOD-GROUP SYSTEM

In England about 28 4% of people are group M, 49 6% group MN and 22% group N, 54 7% are S-positive and 45 3%, S-negative

Grouping for M and N is best done at room temperature when rabbit sera are used Controls of known M, N and MN cells are essential Human anti-M or anti-N sera are rare but not unknown Some seem to be [illegible]

[illegible]

who have had many transfusions, more rarely has the antibody been detected in the sera of women who have given birth to children suffering from haemolytic disease Such antibodies are generally most active at 37°C, they may exist as agglutinins or as incomplete antibodies best detected by the antiglobulin reaction

THE P BLOOD GROUP

[illegible]

of P-negative cells and known P-positive cells are essential. P-positive cells are sometimes classified according to the degree to which they are

value.

Anti-P is a potentially haemolytic antibody, causing weak haemolysis of normal cells, but much stronger haemolysis of trypsinized or P.N.H. cells. On rare occasions its activity extends up to 37°C, and it has then been found to be a cause of transfusion reactions (23).

THE LUTHERAN BLOOD GROUP

About 7·7% of English blood samples are Lutheran-positive (Lu(a+))

examples of an anti-

ome sera seem to have

ature. The sera so far

Albumin and trypsin

ated neither sensitized

THE KELL BLOOD GROUP

globulin test

Kell agglutination should be carried out on fresh blood samples as it seems possible that the antigen deteriorates unusually rapidly on storage (26)

THE LEWIS BLOOD-GROUP SYSTEM

Le^a^ and Le^b^, it is possible to possess

ly not both, at least in adults, i.e., to be

-) About 22% of the population of

on-secretors of A, B or H substances

about 72% are Le(a−b+) and 6% Le(a−b−).

haemolysed

THE DUFFY BLOOD-GROUP SYSTEM

In England about 65 7%

*Fy^b^* and 17 2% *Fy^a^ Fy^a^*).

ost being found in patients

ntibody usually occurs in an

t agglutination in saline or

albumin and treatment with trypsin seems to destroy the antigen (25, 30) However, sensitization may be readily demonstrated by the antiglobulin reaction

THE KIDD BLOOD-GROUP SYSTEM

[illegible]

Race and Sanger (26) mention that stronger reactions may be obtained by carrying out antiglobulin tests on trypsinized cells (29)

"PRIVATE" BLOOD FACTORS (20, 26)

[illegible]

chosen at random It is important, therefore, in attempting to demonstrate an antibody always to include in the panel of test cells those of the actual blood donor(s) (or of the husband) who might have been responsible for the immunization

THE CROSS-MATCHING TEST IN BLOOD TRANSFUSION

It is essential to carry out a cross-matching test between the serum of the recipient and the cells of the donor before every blood transfusion Only if the degree of urgency is such that no delay at all is possible, is it justifiable to break this rule (see later)

The cross-matching test is carried out for two purposes (*a*) to guard against a mistake in ABO or Rh grouping, (*b*) to demonstrate naturally-occurring or immune antibodies in the patient's serum, active against the donor's cells, whose presence could not be anticipated As no single test is capable of disclosing both types of incompatibility satisfactorily, at least two tests have to be carried out as a routine Those recommended are (I) the "saline match" carried out at room temperature and (II) an indirect antiglobulin test, sensitizing the cells at 37°C

Patient's Cells and Serum

Blood from the patient should be obtained if possible a day or two before the transfusion is needed so that his cells may be grouped unhurriedly, with proper controls, and blood of corresponding ABO and Rh group obtained. Serum is preferred to plasma for matching purposes as strong rouleaux formation, which might be a source of confusion, is far less likely to occur

Donor's Blood

A few drops of blood are withdrawn from the transfusion bottle or pilot bottle, using a sterile syringe or Pasteur pipette. The cells should

then be washed in at least one change of saline and a strong (30 to 50%) and a weak (1 to 2%) suspension in saline made. The tests with these cells *should be set up without delay.*

1. *The Saline Cross-Match (Non-Urgent Cases)*

One volume (e.g., 3 drops) of the patient's serum is placed in a 75×8-mm tube and the same volume of a 1 to 2% suspension of donor's washed cells added to it. After mixing, the tube is left undisturbed for 2 hours. Part of the deposit of sedimented corpuscles is then examined microscopically for agglutination, as described on p 193.

The saline cross-match is carried out at room temperature rather than at 37°C. because the naturally-occurring anti-A and anti-B react better at the lower temperature (The agglutinin titre at 37°C. is on the average about half that at 18°C. (17).) Saline rather than bovine albumin is used as a suspending medium, because in albumin anti-A or anti-B activity is reduced to about one-quarter of the activity in saline (17); this may even result in missing ABO incompatibility altogether.

Difficulties: Abnormal Reactions Not Due to ABO Incompatibility

Rouleaux. As the sera may be obtained from severely-ill patients, some rouleaux formation (associated with a raised serum-globulin concentration) is not infrequent. Dextran given to a patient before his serum is obtained for the matching test also causes exaggerated rouleaux formation The differentiation of rouleaux formation from weak agglutination may not be too easy, although experience helps to overcome the difficulty If in doubt, the addition of another volume of saline to the cell suspension on the slide aids differentiation by causing the rouleaux to break up to a greater or lesser extent

The differences between rouleaux formation and weak agglutination may be summarized as follows *Rouleaux formation* affects nearly all the cells present, large clumps are not usually seen but the side-to-side adherence of the cells giving rise to many intermingling columns is a conspicuous feature. *Weak agglutination* affects only a small proportion of cells, large clumps are not formed but the small clumps that are present are widely separated in a field of free unagglutinated cells, the cells adhere to each other at all angles (Figs. 38 and 39).

Cold Agglutinins Occasionally, true agglutination is brought about by cold agglutinins First, ABO incompatibility has to be excluded by showing that the agglutination disappears if the tube is placed at 37°C. and by rechecking the ABO groups of both donor and recipient, secondly, if ABO incompatibility is excluded, steps must be taken to identify the agglutinin It may be a non-specific cold agglutinin if so, it will also agglutinate the patient's washed cells, and other red cells irrespective of blood group, or it may be a specific cold agglutinin

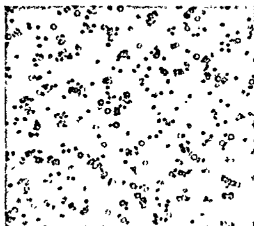


FIG 38 PHOTOMICROGRAPH OF A SUSPENSION OF BLOOD CELLS IN SERUM, SHOWING A MINOR DEGREE OF ROULEAUX FORMATION ×150

The numerous small rouleaux are characteristically relatively evenly spaced throughout the field and do not vary greatly in size

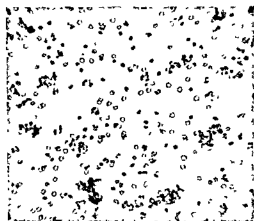


FIG 39. PHOTOMICROGRAPH OF A SUSPENSION OF BLOOD CELLS IN SERUM, SHOWING WEAK AGGLUTINATION ×150

The small agglutinates are more irregularly distributed than the rouleaux and vary more in size There are also more free cells

To face p 202

such as α_1, anti-P, anti-M or anti-Lewis, all of which can be identified by using a panel of cells of known genotype.

Non-specific cold agglutinins may give trouble, even if not present in abnormal amounts, if the temperature of the laboratory is unusually low (e.g., 15°C. or less). Cold agglutination persisting at 20°C. indicates that the serum contains an abnormal antibody such as might be present in acquired haemolytic anaemia. However, if, as is usual, agglutination is abolished at temperatures above 30°C., the donor cells can probably be transfused with safety. If the antibody is a specific one, cells lacking the corresponding antigen should be sought for.

Immune Agglutinins. Agglutinins other than anti-A, anti-B and anti-Rh which give rise to agglutination which persists at 37°C. are rarely met. Anti-M, anti-S, anti-Lutheran, and anti-Le^a are possible types. An attempt should be made to identify the agglutinin by using a panel of cells of known genotype. Indirect antiglobulin tests should also be carried out.

II. THE ANTIGLOBULIN CROSS-MATCH

One drop of a 30 to 50% suspension of washed donor cells is added to 5 to 10 drops of the patient's fresh (not heated) serum in a 75 × 10-mm. tube, mixed and then placed at 37°C. for 2 hours. After having been washed three times in a large volume of saline, the cells are tested with antiglobulin serum for adsorbed antibody. The potency and specificity of the antiglobulin serum must be controlled by testing (*a*) normal cells exposed to normal serum and (*b*) normal cells weakly sensitized with anti-D.

Ideally, the cells should be tested after sensitization with two dilutions of a potent antiglobulin serum, e.g., *1 in 4 and 1 in 64*. Antibodies of the γ-globulin type, e.g., anti-Rh and anti-Kell, react better in highly diluted serum while antibodies such as anti-Le^a and non-specific pathological cold antibodies of high thermal activity react better in strong concentrations.

The antiglobulin test as described above will probably detect any type of antibody likely to be of significance. It is more sensitive and satisfactory than are cross-matching tests using albumin. It has the following advantages over the albumin cross-match: inhibition of agglutination due to prozones caused by large amounts of antibody appears to be most unlikely, antibodies such as anti-Fy^a are detected—these are not revealed at all by the albumin method, and the results are far more easily read.

A further alternative technique is to carry out cross-matching tests at 37°C., using enzyme-treated donor red cells (see p. 120). This is a sensitive method of detecting some types of incomplete antibodies, e.g., anti-Rh and anti-Lewis (*not* anti-Fy^a). It may usefully be used when the results of the antiglobulin cross-match are doubtful or

equivocal in patients suspected on clinical grounds of having formed immune antibodies.

Cross-Matching for Urgent Transfusions

There seems to be no way of cross-matching blood rapidly with perfect safety. However, ABO incompatibility and large (and consequently serious) amounts of incomplete antibody (e.g., anti-Rh) can be detected fairly reliably by the above-described techniques if the cells and serum are allowed to stand in contact for as little as 15 minutes. In urgent cases it is recommended that saline and antiglobulin cross-matches should be set up, as described above. After a minimum of 15 minutes the saline match is read microscopically after centrifuging the tube slowly at 500 to 1,000 r.p.m. for a minute or so. If the cells appear compatible the blood can be released, if the clinician is demanding it, and the antiglobulin cross-match proceeded with. By the time the blood has reached the patient the result of this test should also be known. It should be possible to allow more adequate time for cross-matching tests performed on any subsequent bottles the patient is to receive. A description of alternative cross-matching tests which may be used in urgent cases, including "slide" techniques using albumin, is given by Dodge (10).

If there is absolutel ... "desperate" cases, a bottle of gro ... patient is a woman. ... unless he is known to have been transfused previously when he should receive group-O Rh-negative blood if this is available. If the patient's group is known with absolute certainty, he can, of course, be given blood of his own group.

THE INVESTIGATION OF TRANSFUSION REACTIONS

"Transfusion reactions" vary widely in type and in severity. They have many different causes and considerable experience and sometimes special techniques may be required to elucidate them. Broadly speaking they can be classified in two groups, those produced by serological incompatibility resulting in destruction of the transfused red cells (occasionally the recipient's cells), and those not due to this cause. In the second category are included a wide variety of conditions such as febrile reactions due to pyrogens in the anticoagulant solution or apparatus, urticarial or other reactions due to sensitivity to protein, cardiac embarrassment due to too rapid transfusion, and sudden death due to air embolism. A consideration of cases of this sort is beyond the scope of this book. The laboratory worker is deeply ... in order to establish ... patibility.

The incident should be investigated more or less as follows, depending upon the circumstances:

1. The transfusion should be stopped, or at least brought almost to a standstill, as soon as there is a suspicion that there is something seriously wrong, and that this may be due to some kind of incompatibility leading to haemolysis

2. A sample of venous blood should be collected from a vein well away from the transfusion site. This blood should be withdrawn slowly into a dry syringe; part should be delivered into a bottle containing heparin or a small volume of 3·8% sodium citrate solution and part placed in a plain bottle and allowed to clot. Every care should be taken to avoid haemolysis during collection Instructions should be given that the next specimen of urine passed by the patient should be saved and the blood bottle preserved if not yet disconnected from the patient. Enquiry should be made as to how the blood has been treated, whether it has been warmed and how long it has been out of the refrigerator, etc.

3. On return to the laboratory part of the patient's heparinized or citrated blood should be immediately centrifuged and the supernatant plasma inspected If there is no evidence of free haemoglobin or obvious increase in bilirubin, it is not likely that there has been any serious degree of haemolysis. The presence or absence of oxyhaemoglobin, methaemalbumin or increased bilirubin in the plasma naturally depends on the rate the blood was being transfused before the sample was taken and the rate of haemolysis

A small drop of the recipient's blood should be placed on a slide and allowed to spread out under a cover-glass If incompatible blood has been administered and not yet entirely destroyed, small clumps agglutinated (donor) cells may be visible A direct antiglobulin should also be carried out on the recipient's blood The test probably be positive if incompatible donor cells are still circulating relatively large numbers

A sample of the donor's blood should also be centrifuged and th supernatant plasma inspected for haemolysis If there is any suggestion that the blood was infected, it should be cultured The age of the blood should be ascertained

4. If it seems possible that the haemolysis was due to serological incompatibility, the next step is to try to find the cause This should be done in several stages

(a) Blood from the donor and the recipient should be re-grouped, and saline and antiglobulin cross-matching tests performed again They may show straightaway that some gross error has been committed. If the results of these tests are negative or doubtful, a cross-matching test using trypsinized or papainized donor cells should be set up and incubated at 37°C.

Pre-transfusion serum should be used, if available, if serum

equivocal in patients suspected on clinical grounds of having formed immune antibodies.

Cross-Matching for Urgent Transfusions

There seems to be no way of cross-matching blood rapidly with perfect safety. However, ABO incompatibility and large (and consequently serious) amounts of incomplete antibody (e g, anti-Rh) can be detected fairly reliably by the above-described techniques if the cells and serum are allowed to stand in contact for as little as 15 *minutes* In urgent cases it is recommended that saline and antiglobulin cross-matches should be set up, as described above. After a *minimum* of 15 *minutes* the saline match is read microscopically after centrifuging the tube slowly at 500 to 1,000 r.p.m. for a minute or so. If the cells appear compatible the blood can be released, if the clinician is demanding it, and the antiglobulin cross-match proceeded with. By the time the blood has reached the patient the result of this test should also be known. It should be possible to allow more adequate time for cross-matching tests performed on any subsequent bottles the patient is to receive. A description of alternative cross-matching tests which may be used in urgent cases, including "slide" techniques using albumin, is given by Dodge (10).

If there is absolutely no time for a cross-match, e.g., in "desperate" cases, a bottle of group-O Rh-negative blood should be issued, if the patient is a woman. If a man, group-O Rh-positive blood can be given unless he is known to have been transfused previously when he should receive group-O Rh-negative blood if this is available. If the patient's group is known with absolute certainty, he can, of course, be given blood of his own group.

THE INVESTIGATION OF TRANSFUSION REACTIONS

"Transfusion reactions" vary widely in type and in severity They have many different causes and considerable experience and sometimes special techniques may be required to elucidate them Broadly speaking they can be classified in two groups, those produced by serological incompatibility resulting in destruction of the transfused red cells (occasionally the recipient's cells), and those not due to this cause. In the second category are included a wide variety of conditions such as febrile reactions due to pyrogens in the anticoagulant solution or apparatus, urticarial or other reactions due to sensitivity to protein, cardiac embarrassment due to too rapid transfusion, and sudden death due to air embolism A consideration of cases of this sort is beyond the scope of this book The laboratory worker is deeply concerned, however, in the investigation of reactions in order to establish first, whether a particular reaction is due to serological incompatibility, and secondly, to find out why this has occurred

The incident should be investigated more or less as follows, depending upon the circumstances:

1. The transfusion should be stopped, or at least brought almost to a standstill, as soon as there is a suspicion that there is something seriously wrong, and that this may be due to some kind of incompatibility leading to haemolysis.

2. A sample of venous blood should be collected from a vein well away from the transfusion site. This blood should be withdrawn slowly into a dry syringe, part should be delivered into a bottle containing heparin or a small volume of 3·8% sodium citrate solution and part placed in a plain bottle and allowed to clot Every care should be taken to avoid haemolysis during collection Instructions should be given that the next specimen of urine passed by the patient should be saved and the blood bottle preserved if not yet disconnected from the patient. Enquiry should be made as to how the blood has been treated; whether it has been warmed and how long it has been out of the refrigerator, etc.

3. On return to the laboratory part of the patient's heparinized or citrated blood should be immediately centrifuged and the supernatant plasma inspected If there is no evidence of free haemoglobin or obvious increase in bilirubin, it is not likely that there has been any serious degree of haemolysis The presence or absence of oxyhaemoglobin, methaemalbumin or increased bilirubin in the plasma naturally depends on the rate the blood was being transfused before the sample was taken and the rate of haemolysis.

A small drop of the recipient's blood should be placed on a slide and allowed to spread out under a cover-glass If incompatible blood has been administered and not yet entirely destroyed, small clumps of agglutinated (donor) cells may be visible A direct antiglobulin test should also be carried out on the recipient's blood The test will probably be positive if incompatible donor cells are still circulating in relatively large numbers

A sample of the donor's blood should also be centrifuged and the supernatant plasma inspected for haemolysis. If there is any suggestion that the blood was infected, it should be cultured The age of the blood should be ascertained.

4. If it seems possible that the haemolysis was due to serological incompatibility, the next step is to try to find the cause This should be done in several stages

(a) Blood from the donor and the recipient should be re-grouped, and saline and antiglobulin cross-matching tests performed again. They may show straightaway that some gross error has been committed If the results of these tests are negative or doubtful, a cross-matching test using trypsinized or papainized donor cells should be set up and incubated at 37°C.

Pre-transfusion serum should be used, if available; if serum

withdrawn after transfusion is employed, incompatibility may be missed because the causal antibodies may have been completely absorbed *in vivo* by the donor's blood. Similarly, pre-transfusion corpuscles should be used for re-grouping the patient if they are available. Confusion may arise if blood is used which has been withdrawn from the patient *after* an incompatible transfusion. Under these circumstances it is possible for a group-A subject, transfused with B cells, to appear to belong to group AB, or an Rh-negative person, transfused with Rh-positive cells, to appear to be Rh-positive, because the recipient's and donor's cells may be present together.

(*b*) If the cross-matching tests are clearly negative, it is unlikely, although not impossible, that the haemolytic reaction is due to haemolysis of the donor's corpuscles. The next step is to consider the possibility that haemolysis of the patient's corpuscles has taken place, perhaps due to immune anti-A (or anti-B) being transfused in group-O blood given to group-A (or -B) recipients. The anti-A (or anti-B) titre of the transfused plasma should be ascertained and tests carried out for anti-A (or -B) haemolysin and for incomplete antibodies (see p. 190) In paroxysmal nocturnal haemoglobinuria the patient's red cells are extraordinarily sensitive to isohaemolysin. Even small volumes of plasma may precipitate a haemolytic episode (7).

If group-O blood has, in fact, been given to a group-A or -B recipient the osmotic fragility of the recipient's blood should be measured and blood films stained. An increase in osmotic fragility and the presence of spherocytes are pointers to the haemolytic reaction being due to the transfusion of immune anti-A or anti-B (11).

(*c*) It may be possible to determine the cause of the haemolytic reaction by measuring the survival of the transfused corpuscles by means of the differential-agglutination method (p. 130) By the use of anti-A, anti-B, anti-M or anti-Rh sera it is often possible to demonstrate unequivocally that the transfused corpuscles are present in the expected numbers (approximately 300,000 to 400,000 per c mm. in adults per 500 ml. of citrated blood transfused) or that far fewer than expected are present, or that all have disappeared By counting the total number of red cells as well as the number of the transfused red cells it may even be possible to prove without doubt that the haemolysis has been due entirely to destruction of the patient's own cells. A survival study may also demonstrate minor or " silent " degrees of blood destruction of insufficient rapidity to cause haemoglobinaemia or a significant rise in plasma-bilirubin concentration

(*d*) If the patient is not seen until after the incident and if no donor blood is available, the cause of an incompatible transfusion may be revealed by an increase in titre of an iso-antibody present in the patient's serum, e.g., a many-fold rise in anti-A in a group-B subject, which reaches a peak 1 to 2 weeks after transfusion, is a clear indication that the recipient had received group-A blood

The introduction of the technique of labelling red cells with radiochromium (^{51}Cr) has provided a further valuable tool for the investigation of obscure transfusion reactions. In a patient who has recovered from a transfusion reaction, it is now possible to tag a few ml. of the blood believed to be incompatible and to follow accurately (without harm to the patient) its survival after intravenous injection and thus to confirm or refute its incompatibility (23)

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CHAPTER 12

MISCELLANEOUS TESTS

ESTIMATION OF THE ERYTHROCYTE SEDIMENTATION RATE (E.S.R.)

ALTHOUGH an empirical test, the estimation of the erythrocyte sedimentation rate has been widely used in clinical medicine Many methods for its measurement have been devised (11, 17), differing in respect of the anticoagulant used, the volume of blood employed, the dimensions of the tube in which the measurement is carried out, the time allowed for sedimentation to take place, and the method of recording the results. Two methods will be described

(1) Method of Westergren (22)

The Westergren tube is a straight glass tube 30 cm. in length and 2·5 mm. in diameter, it is calibrated in mm from 0 to 200 mm

Venous blood is diluted with a one-fifth volume of 3 8% trisodium citrate The sample is well mixed and the blood is then drawn up into the Westergren tube to the 200-mm mark The tube is placed exactly vertical and left undisturbed for 60 minutes The height of the clear plasma above the upper limit of the column of sedimenting red cells is then read to the nearest mm This figure in mm per hour is the E S R. A second reading can be taken at the end of a further 60 minutes

(2) Method of Wintrobe and Landsberg (24)

In this method dry mixed ammonium and potassium oxalates (p 219) are used as the anticoagulant at a concentration of 2 mg per ml The tube used is a standard Wintrobe haematocrit tube The sample is well mixed and the tube then filled to the 100-mm. mark The height of the column of supernatant plasma in mm is then read at the end of 60 minutes and recorded as the E S R Finally, the haematocrit tube may be centrifuged for 30 minutes at 3,000 r p m and the packed cell volume measured The sedimentation rate can then be "corrected" for anaemia using Wintrobe and Landsberg's (24) chart and values obtained corresponding with packed cell volumes of 47% and 42% in men and women, respectively

Normal Range

Men (Westergren's method)	3 to 5 mm. in 1 hour. 7 to 15 mm. in 2 hours.
(Wintrobe and Landsberg's method)	0 to 9 mm in 1 hour.
Women (Westergren's method)	4 to 7 mm. in 1 hour. 12 to 17 mm. in 2 hours.
(Wintrobe and Landsberg's method)	0 to 20 mm. in 1 hour.

MECHANISM OF ERYTHROCYTE SEDIMENTATION

The phenomenon of erythrocyte sedimentation has been exhaustively investigated (11, 21)

The rate of fall of the red cells depends very largely upon their power of forming rouleaux, large clumps which sediment much more rapidly than single cells. This property is mainly controlled by the concentrations of fibrinogen and to a lesser extent of globulin in the plasma. Defibrinated blood sediments extremely slowly, not more than 1 mm. per hour, unless the serum-globulin concentration is raised or there is an unusually high globulin-albumin ratio. Red-cell factors, however, also play a part (12, 19).

[illegible]

cases serial readings are useful Probably the most satisfactory method of [illegible]

[illegible] rate of sedimen- [illegible] no anticoagulant [illegible] A rise in tempera- [illegible] deviation of the [illegible] be 2 5 mm. or greater, and the height not less than 100 mm. These and other modifying factors are considered in full by Ham and Curtis (11) and reviewed by Nichols (17) A poor delineation of the upper layer of red cells, so-called " stratified sedimentation ", has been attributed by Stephens (20) to the presence of many reticulocytes

The relationship between E S R and plasma viscosity is considered by [illegible] -neral increase in parallel, but when [illegible] ate of fall of the rouleaux becomes [illegible] outweighing the enhancing effect of the colloid or protein on rouleaux formation

Westergren and Wintrobe and Landsberg's Methods Compared

[illegible]

give normal readings when the results with the Westergren method are definitely abnormal (7, 9). Goldberg, Glynn and Bywaters (9), who observed that heparinized blood as well as oxalated blood might sometimes sediment unexpectedly slowly, found that the addition of sodium citrate increased the rate of sedimentation.

Wintrobe and Landsberg's method has the theoretical advantage of allowing correction for anaemia should this be present. However, there is doubt [illegible]

comparable with that of a normal blood diluted with plasma to the same [illegible]

purposes

THE PAUL-BUNNELL TEST FOR HETEROPHILE ANTIBODY

The presence of anti-sheep-cell haemagglutinins at unusually high titres in the sera of patients suffering from glandular fever (infectious mononucleosis) was described in 1932 by Paul and Bunnell (18). Absorption methods for differentiating between the glandular-fever antibodies and the naturally occurring ones, and those of serum sickness, have since been widely used (3). The technique described below is based on that of Barrett (1).

The following reagents are required

1. 1 ml. of the patient's serum, previously inactivated by heating at 56°C for 30 minutes.
2. Guinea-pig kidney emulsion (see p. 214).
3. A 2% autoclaved ox red-cell suspension (see p. 214).
4. A freshly prepared 0.4% saline suspension of washed sheep red cells. The sheep blood should preferably be not more than 7 days old.

METHOD

Three 0·25-ml. volumes of patient's inactivated serum are delivered into three small tubes, *A*, *B* and *C*. 1·0 ml. of saline is added to tube *A*, 0·75 ml. of saline and 0·3 ml. of guinea-pig kidney emulsion are added to tube B, and 1 ml. of ox-cell suspension is added to tube *C*. The contents of the three tubes are mixed and then placed in the refrigerator at 4°C. *for at least 2 hours or overnight. The tubes are then centrifuged* and the supernatants retained. One in five dilutions in saline of unabsorbed serum and of the serum absorbed with guinea-pig kidney and ox red cells, respectively, are thus obtained.

A Screening Test

0·25 ml. of a 0·4% sheep-cell suspension is added to 0·25 ml. of *the serum absorbed with guinea-pig kidney (from tube B). After* leaving the tube at room temperature for 15 minutes, it is centrifuged for 2 minutes at 1,000 r.p.m. An attempt is then made to re-suspend the deposit by gently tapping the tube If the deposit re-suspends easily and there is no macroscopic agglutination when the contents of the tube are viewed with a concave mirror, the whole test is considered to be negative. *If there is agglutination, a quantitative test should be* carried out

The Quantitative Test

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0· [illegible]

co[illegible] of the 0 4% sheep-cell suspension are then added to each tube, giving final serum dilutions of from 1 in 10 (tube 1) to 1 in 2,560 (tube 9). After mixing their contents the tubes are incubated for 2 hours at 37 C before the results are read A standardized method for reading the end-point should be adopted. Macroscopic reading using a concave mirror is recommended (p. 106) Serum known to contain glandular-fever antibodies should be absorbed and titrated as a control for the potency of the absorbents and the agglutinability of the sheep red cells.

The following figures are given as examples of typical results

Unabsorbed serum, end point tube 7, titre 640.
Guinea-pig kidney absorbed serum, end point tube 7; titre 640
[illegible] tube 4 titre 80

Suc[illegible] fever, the antibody being [illegible] artially) absorbed by the ox cells The naturally occurring antibody is absorbed by guinea-pig kidney, but not by ox cells, and that of serum sickness is absorbed by both reagents

Unabsorbed serum, end point tube 3, titre 40
Guinea-pig absorbed serum, no agglutination in tube 1.
Ox-cell absorbed serum, end point tube 3; titre 40

This is a normal result, and the screen test would have been negative.

The absorbing power of the guinea-pig kidney emulsion and the ox cells must be tested with known positive and negative sera from time to time Barrett (1) stated that each will keep for at least 6 months if stored at 4°C.

The antibodies normally ... of the Forssman type, i e spread in animal tissues ...

Technical Factors Affecting Results

Temperature. The heterophile antibody of glandular fever is a warm antibody and there is no point in exposing the cell-serum suspensions to temperatures below 37°C Indeed, agglutination due to cold antibodies and not to the glandular-fever antibody may cause confusion if the titrations are placed at 4°C before the results are read (26)

Varying Sensitivity of the Sheep Red Cells A cause of difficulty in serial studies is the comparatively wide variation in sensitivity between one sample of sheep red cells and the next Zarofonetis and Oster (25) tested 24 sera with the red cells from 24 different sheep They found the titres given by the most sensitive cells to be from 4 to 16 times those given by the least sensitive cells

Interpretation of Results

The Paul-Bunnell test is generally looked upon as a most useful aid ...

Antibodies are often present as early as the fourth to sixth day of the disease (2) and are almost always found by the 21st day (14) They disappear as a rule within 4 to 5 months (2)

Two points need further consideration: (a) the frequency of negative reactions in glandular fever; and (b) the sheep-cell agglutinin titre in health and the specificity of positive reactions

that they may be missed or may produce anomalous reactions when associated with the naturally occurring antibody at similar titres. For all the above reasons it is difficult to state categorically that any particular patient has not or will not produce antibodies

The limits of the titre of heterophile antibodies present in health are ill-defined, largely because of differences in the techniques used to demonstrate them. *Fortunately, this is of little consequence, because in the diagnosis of glandular fever it is only the antibody not absorbed by guinea-pig kidney which is of significance*

In connection with the possibility of false positive reactions, Barrett's (1)

diagnostic

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concentrations giving the typical reactions of glandular fever are ever found in other diseases uncomplicated by glandular fever In particular, the

presence of glandular-fever antibody (15)

PREPARATION OF GUINEA-PIG KIDNEY SUSPENSION AND HEATED OX RED-CELL SUSPENSION (after Barrett (1))

Ox Red-Cell Suspension. and a 30% suspension is autoclaved at ure for 20 minutes When

cool, the suspension is strained through muslin and its packed cell volume estimated. The packed cell volume is then adjusted to 20% with saline, and an equal volume of 1% phenol-saline added to give a 10% suspension. It is diluted to a 4% suspension before use.

The ability of the suspension to absorb the glandular-fever antibody must be tested with known positive sera from time to time

SPECTROSCOPIC EXAMINATION OF BLOOD FOR METHAEMOGLOBIN AND SULPHAEMOGLOBIN

Method. Blood is diluted 1 in 5 or 1 in 10 with water and then centrifuged. The clear solution is examined preferably in daylight in a glass cell or tube, using a hand spectroscope It is important that the greatest possible depth or concentration of solution consistent with visibility should be examined, and that a careful search should be made (with varying depths or concentrations of solution) for absorption bands in the red part of the spectrum (620 to 630 mμ) If bands are seen, the solution should be treated with a drop of yellow ammonium sulphide. A band due to methaemoglobin will then disappear, if sulphaemoglobin is present, its band persists For comparison, laked blood may be treated with potassium ferricyanide solution, which will cause the formation of methaemoglobin A sample of sulphaemoglobin may be prepared from blood (10 ml of 1 in a 100 dilution) by adding to it phenylhydrazine hydrochloride solution (0 1 ml. of a 0 1% solution) and a drop of water saturated with hydrogen sulphide. The unknown and the known pigments may then be compared in a reversion spectroscope

The absorption band in the red due to methaemoglobin is at the wavelength 630 mμ (cf, methaemalbumin at 624 mμ) (Fig 40)

Methaemoglobin and Sulphaemoglobin are formed intracellularly, they are not found in plasma except under very exceptional circumstances (e.g., when intense methaemoglobin or sulphaemoglobin formation is associated with intravascular haemolysis)

SPECTROSCOPIC EXAMINATION OF PLASMA FOR METHAEMALBUMIN

The plasma is freed from suspended cells and platelets by centrifuging at 3,000 r p m for 15 to 30 minutes It is then viewed in bright daylight with a hand spectroscope using the greatest possible depth of plasma consistent with visibility Methaemalbumin gives a rather weak band in the red (at 624 mμ) (Fig 40) As oxyhaemoglobin is usually present as well, its characteristic bands in the yellow-green may also be visible The position of the methaemalbumin absorption band in the red can readily be differentiated from that of methaemoglobin by means of a reversion spectroscope

Presumptive evidence of the presence of small quantities of methaemalbumin, giving an absorption band too weak to recognize, ca.

obtained by converting the pigment to an ammonium haemochromagen which gives a more intense band in the green (Schumm's test).

SCHUMM'S TEST

Method. The serum (or plasma) is covered with a layer of ether. A one-tenth volume of saturated yellow ammonium sulphide is added

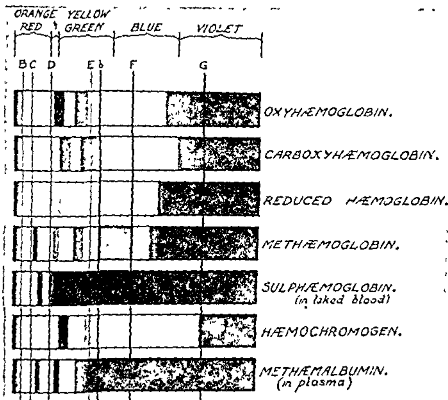


FIG 40 ABSORPTION SPECTRA OF DERIVATIVES OF HUMAN HAEMOGLOBIN
[illegible] *C* at 656 3 mμ, *D* at 589 mμ, and *G* at 430 8 mμ
[illegible] nel Whitby and Dr C J C [illegible] l, London

and mixed with the serum, which is then viewed with a hand spectroscope. If methaemalbumin is present, a relatively intense narrow absorption band will be seen in the green (at 558 mμ) (Fig 40)

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CHAPTER 13

APPENDICES

APPENDIX 1: PREPARATION OF CERTAIN REAGENTS

Chapter 1

Anticoagulants

Ammonium and potassium oxalate mixture

Ammonium oxalate	1 2 g
Potassium oxalate	0 8 g
Distilled water	to 100 ml.

0 4 ml. (or 0 2 ml.) of the solution, containing 8 mg (or 4 mg.) of the mixed salts, is delivered into small bottles or tubes marked at the 4-ml (or 2-ml.) level. The bottles or tubes are then placed in an oven at a temperature not exceeding 80 C for the oxalate solution to dry

Sequestrene (di-sodium salt of ethylenediamine tetra-acetic acid (EDTA))

(*a*) *Dry salt* A 10% (w/v) solution is made in distilled water 0 08-ml. volumes are then delivered by pipette into small bottles or tubes marked at the 4-ml. level. (If desired, a Pasteur pipette calibrated to deliver 50 drops per ml. may be used to deliver 4 drops of the solution into each bottle or tube.)

Sodium citrate ($Na_3C_6H_5O_7$, $2H_2O$)

A 3 8% (w/v) solution is made in distilled water Convenient (e g, 10-ml.) volumes should be distributed into small bottles and sterilized by autoclaving (2).

Heparin

Powdered heparin is dissolved in distilled water at a concentration of 4 mg. per ml. 0 25 ml of this solution (containing 1 mg of heparin) is delivered into tubes and bottles which are then placed in an incubator at 37°C for their contents to dry 1 mg. of heparin is sufficient to prevent the coagulation of 5 to 10 ml of blood for at least 24 hours

Chapter 2

Thomson's Inorganic Grey Solution

Chrome alum ($Cr_2(SO_4)_3$, K_2SO_4, $24H_2O$ (Analar))	16 67 g.
Copper sulphate ($CuSO_4$, $5H_2O$ (Analar))	33 33 g.
Cobalt ammonium sulphate ($CoSO_4(NH_4)_2SO_4$, $6H_2O$ (recryst))	39 50 g.
Potassium dichromate ($K_2Cr_2O_7$ (Analar))	0 12

The salts are dissolved in distilled water at room temperature and the resultant solution allowed to age for 6 weeks in a glass-stoppered bottle. The freshly prepared solution is slightly pink in colour, but by the time ageing is completed, it becomes an even grey. The pH should then be 2·64.

The solution can be kept at room temperature for many months without deterioration. It can be used as a permanent standard in haemoglobinometry (see p. 29)

Gibson and Harrison's Artificial Haemoglobin Standard

Chromium potassium sulphate ($CrK(SO_4)_2$, $12H_2O$) ..	11·61 g.
Cobaltous sulphate (anhydrous) ($CoSO_4$)	13·1 g.
Potassium dichromate ($K_2Cr_2O_7$)	0 69 g.
Distilled water	to 500 ml.

1·8 ml. of N-sulphuric acid are added to the dissolved salts and the mixture is heated to boiling. After boiling for 1 minute, the solution is cooled and the volume made up to 1 litre with distilled water.

The chromium potassium sulphate crystals must be free from any signs of

100 ml. (based on iron determinations) when used as described on p 31.

Chapter 8

Formulae of Phosphate and Citrate-HCl Buffers Used in Elution of Antibodies

Kidd's Method

0 066 M-phosphate buffer, pH 5 7	
KH_2PO_4 (0 908%)	50 ml
0 066 N-NaOH	3 72 ml
0 1 M-citrate-HCl buffer, pH 3 2	
$Na_3C_6H_5O_7$, $1\frac{1}{2}H_2O$ (2 631%)	45 ml
0 1 N-HCl	55 ml

Mitchell's Method

Dialysis buffers	*0 5 M-KH_2PO_4*	*0 1 N-KOH*	*Distilled water to*
I 0 02, pH 5 4	38 25 ml.	4 3 ml	1 litre
I 0 04, pH 5 4	76 5 ml.	8 6 ml	1 litre
I 0 06, pH 5 6	114 75 ml	12 9 ml	1 litre

Eluting buffer	*NaH_2PO_4, $2H_2O$*	*Na_2HPO_4*
I 0 15, pH 5 7	2 7%	2 4%
	(15 parts)	(1 part)

Iso-osmotic phosphate buffer, pH 7 4	
NaH_2PO_4, $2H_2O$ (2 34%)	18 ml
Na_2HPO_4 (1 63%)	82 ml

CHAPTER 10

Sodium citrate.
See p 219.

Calcium chloride

A 0 025M solution is made by dissolving 2 77 g of the anhydrous salt in 1 litre of distilled water.

Aluminium-hydroxide gel (1)

50 ml. of ammonia (Sp. G. 0 88) are diluted with 50 ml of distilled water and then poured into 600 ml of distilled water at 63°C. containing 22 g of ammonium sulphate. The temperature is brought rapidly to 58°C and the

0 88 ml. of diluted ammonia, subsequent washings are carried out with plain distilled water. After the washing has been completed the final precipitate is *suspended in the least amount of water that is required to make a gelatinous* suspension that can be pipetted

For use as an absorbent for prothrombin, etc, one drop (0 025 ml) is added to 1 ml of plasma The mixture is incubated at 37°C for 5 minutes and then centrifuged A one-stage prothrombin time carried out on the supernatant should exceed 60 seconds, if it does not, the absorption must be repeated

Veronal buffer, pH 7 35

0 1 M-sodium diethyl barbiturate ($C_8H_{11}O_3N_2Na$)	570 ml
0 1 N-HCl	430 ml
Sodium chloride	5 67 g

Before use the buffer is diluted with an equal volume of 0 9% NaCl

APPENDIX 2: PREPARATION OF GLASSWARE

Flask for the Defibrination of Blood

A 100-ml conical flask is provided with a central glass rod on to the bottom end of which are fused pieces of glass capillary (Fig 41) The rod is kept

from small clots

Glass-Capillary Automatic Pipettes

Small automatic pipettes (3) made to deliver small volumes, e g, 0 05

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Pra ... these pipettes can be effectively used, for in unpractised hands fluid may be lost by being allowed to spill over into the outer tube.



FIG 41 FLASK FOR DEFIBRINATING 10 TO 50 ML OF BLOOD
The central glass rod has had some small pieces of drawn-out glass capillary fused to its lower end

Siliconed Glassware

A 10% (v/v) solution of silicone, M 441 (I.C.I.), is made in petroleum ether. The clean glassware or syringes to be coated are immersed in the fluid and allowed to drain dry. (It is advisable to wear rubber gloves and to prepare the apparatus in a fume cupboard provided with an exhaust fan.) The coated glassware is then allowed to soak overnight in distilled water, rinsed in a change of distilled water and finally allowed to dry in an incubator.

APPENDIX 3: METHODS OF CLEANING SLIDES AND APPARATUS

New Slides

These should be placed in dichromate cleaning fluid for at least 48 hours. (The cleaning fluid consists of potassium dichromate, 20 g, dissolved in 100 ml of distilled water, to which is then added 900 ml of concentrated sulphuric acid.) The treated slides are well washed in running water, rinsed in distilled water and stored until used in 95% ethanol. They should be dried with a clean linen cloth and carefully wiped free from dust before they are used.

Dirty Slides

When discarded, they should be placed in an antiseptic such as a 2% solution of Chloros (I.C.I.). They should then be washed in running tap water and placed in a commercial detergent solution, e.g. Brylvanz (G. T. Gurr), and boiled for 20 minutes. After a wash in tap water, the slides should be rinsed in 5% hydrochloric acid and then washed in hot running tap water. Finally, they should be rinsed in distilled water before being dried with a clean linen cloth.

Chemical Apparatus and Glassware

The apparatus should be washed in running tap water and then boiled in a detergent solution, rinsed in acid and washed in hot running tap water, as described above. Alternatively, the apparatus can be soaked in dichromate-sulphuric acid mixture.

APPENDIX 4: THE STERILIZATION OF SYRINGES AND NEEDLES

Syringes and Needles

Syringes of 10-ml, 20-ml or 30-ml capacity and sizes 19 or 20 (S.W.G.)
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...ould. Needles should be well cleaned with a pledget of wool on a stick or wire, as in a throat swab, and the lumen cleared with a wire stilette.

Needles and Rubber Tubing

Needles with olive mounts and of gauge 16, as used for giving blood transfusions, are suitable for use when relatively large (e.g., 50-ml) volumes

of blood are required About 5 ...

100 C.

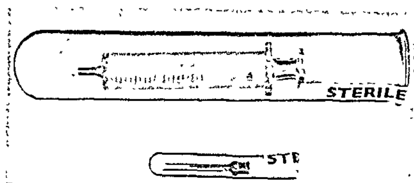


FIG 42 STERILE SYRINGE AND NEEDLE
It is convenient to sterilize syringe and needle separately

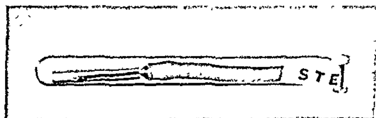


FIG 43 STERILE NEEDLE AND TUBING FOR THE COLLECTION OF 20 TO 50 ML OF BLOOD
The needle has an olive mount and should be of about 16 S W G

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